INHERITANCE OF ORGANELLE GENOMES IN CITRUS

By

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The citrus nuclear genome has been investigated for a number of years. In contrast, little is known about the citrus organelle genome. Restriction fragment length polymorphisms (RFLPs) were the method used in this research for the characterization of citrus organelle inheritance. Six mitochondrial and two chloroplast heterologous probes were used to study the organelle inheritance in two different sources of materials--the F1 progeny of a citrus intergeneric sexual cross and the products of eight different citrus protoplast fusions.

The most common mode of organelle inheritance in angiosperms is uniparental-maternal; however the sexual cross produced unexpected results in terms of apparent biparental inheritance of mitochondria for three

mitochondrial genes: *atpA, coxII* and *coxIII*. The same F1 hybrids presented a strict maternal inheritance of the mitochondrial *coxI* and *26SrRNA*. This raised the possibility that there was a nuclear influence over the mitochondrial genome organization, which has been ignored in previous studies where the biparental inheritance of organelles has been reported. A maternal inheritance of the chloroplast *cytf* and *petD* was observed in the same cross.

All the cybrids and somatic hybrids in this work inherited their mitochondrial genome from the embryogenic fusion partner (callus or cell suspension). In some of the combinations, non-parental bands were observed among the mitochondrial configurations. In contrast, the cybrids and somatic hybrids inherited plastid DNA from either the embryogenic or the nonembryogenic (leaf) fusion partner. The relative abundance of organelle DNAs in the embryogenic and leaf cells may be the basis of these inheritance patterns. Both sexual crosses and somatic cell fusions can therefore produce novel combinations of organelle genes in citrus.

CHAPTER 1
INTRODUCTION

Citrus

"*Citrus* fruits have been cultivated and enjoyed for over 4,000 years" (Davies and Albrigo, 1994, p. vii). *Citrus* includes many commercially important fruit species such as sweet orange (*C. sinensis*), mandarin (*C.reticulata*), lemon (*C.limon*), grapefruit (*C. paradisi*), and it is grown in over 100 countries in tropical and subtropical areas, between the latitudes 40°N to 40°S.

The world produces around 70 million tons of citrus fruits/year. Brazil is now the largest producer of citrus worldwide having an industry oriented towards production of oranges for processing. This country is followed closely by the United States of America, the second largest producer of citrus and the largest producer of grapefruit (Davies and Albrigo, 1994).

Most of the citrus grown today originated by chance during seedling selections or bud mutations of existing cultivars and not from breeding programs (Grosser and Gmitter, 1990). The lack of success in breeding programs is

due to several aspects of the reproductive biology of citrus like heterozygosity and inbreeding depression, pollen and ovule sterility, apomixis, nucellar polyembryony and juvenility (Chapter 2). According to Grosser and Gmitter (1990): "future success will be achieved by combining conventional breeding methods with new biotechnologies" (p.344).

Somatic Hybridization

Somatic hybridization via protoplast fusion is a useful technique for the production of novel plants which cannot be obtained by conventional breeding methods. In 1975, Vardi et al. reported the isolation and culture of sweet orange protoplasts. Ohgawara et al. (1985) produced the first citrus somatic hybrid plants, followed by Grosser et al. (1988) producing the first somatic hybrid plants from sexually incompatible parents (reviewed by Grosser and Gmitter, 1990). In these more than 20 years, different methodologies were developed, and the protoplast fusion technique is an important tool for citrus breeding programs. Another possible application for somatic hybridization is the direct fusion-mediated transfer of cytoplasmic encoded traits (Chapter 2).

Organelle Genetics

In the 1960s, the study of organelle genetics gained importance with the discovery that plastids and mitochondria contain their own genomes. In the 1970s, the study of organelle heredity became a mature field. Much was learned about organelle DNA, the genes encoded by plastids and mitochondria, and the protein synthesizing apparatus of these organelles (Gillham, 1994).

Organelle inheritance is a very complex phenomenon and ever since the first demonstrations of maternal inheritance of plastid mutations, numerous exceptions have been discovered. About one-third of all higher plant genera at least occasionally, inherit plastids biparentally (Chapter 2). Unless very low probabilities of paternal or biparental inheritance can be detected, researchers may incorrectly assume strict maternal inheritance of organelles.

When making wide crosses, plant breeders should be aware of the nature of organelle transmission, since biparental transmission could have a lasting effect on the final product. Especially significant would be cytoplasmic male sterility genes, because any paternal transmission could lead to undesirable results (Soliman et al., 1987). Indeed, even a very small amount of paternal leakage has to be taken into account in the studies of relatedness between

individuals that assume the maternal inheritance of organelles. Also, regular biparental inheritance of organelles could represent a method for achieving cytoplasmic hybridization.

Research Objectives

The objective of this work was to analyze the organelle composition of plants recovered from eight different protoplast fusion experiments performed at the CREC (UF/Lake Alfred/FL), in Dr. Grosser lab. Fusion products were obtained when combining embryogenic culture-derived protoplasts with leaf-derived protoplasts. Two of these combinations produced tetraploid plants, with morphology intermediary between the two parents, characteristic of somatic hybrids. The other six analyzed combinations produced diploid plants with the leaf parent morphology and are probably cybrids (Chapter 2).

Restriction endonuclease technology, which allows the identification of restriction fragment length polymorphisms (RFLPs) has been found useful for studying organelle inheritance in hybrid plants. In this work, six mitochondrial and two chloroplast heterologous probes were used for organelle inheritance characterization.

Before proceeding to the organelle analysis of the somatic hybrids and cybrids, we decided to check the

organelle inheritance in a citrus sexual cross, to test the assumption of maternal inheritance. The citrus nuclear genome has been investigated for a number of years. In contrast, little is known about the citrus organelle genome. Dr. Gmitter supplied us with an intergeneric sexual cross between LB 1-18 (a hybrid of *Citrus reticulata* Blanco cv. 'Clementine' x *C. paradisi* Macf. cv. 'Duncan') and *Poncirus trifoliata* (L.)Raf.

Citrus and *Poncirus* belong to the family Rutaceae. *Poncirus* consists of two trifoliate species, *Poncirus trifoliata* and *P. polyandra*. *P. trifoliata* is a deciduous tree native to cold regions in Central China. It is an important rootstock in some parts of the world because it is cold hardy and it is resistant to the Tristeza virus, citrus nematode (*Tylenchulus semipenetrans*) and *Phytophthora*. For these reasons it has also been used as a parent in citrus rootstock breeding. *Citrus* and *Poncirus* have different morphologies. However, both genera are diploid (2n=18) and they are sexually compatible (Davies and Albrigo, 1994).

The sexual cross produced unexpected results, in terms of a possible biparental inheritance of mitochondria for three mitochondrial genes: *atpA, coxII* and *coxIII*. We thought citrus would be one more exception to the uniparental inheritance of organelles, but further analyses revealed that the same F1 hybrids presented apparently a strict maternal

inheritance of the mitochondrial *coxI* and *26SrRNA*. So, added to the original objective of analyzing the organelle inheritance of citrus somatic hybrids and cybrids, this intergeneric cross provided us with some extra exciting questions in terms of the mitochondria genome organization and the nuclear influence over the organelle inheritance in citrus.

CHAPTER 2
LITERATURE REVIEW

Organelles

According to Gillham (1994), one of the major features distinguishing eukaryotic and prokaryotic cells is that eukaryotic cells are populated with a variety of subcellular organelles. Of the many types of organelles found in the cell, chloroplast and mitochondria are described by this author as very distinctive, in possessing their own genomic system. Mitochondria are the principal source of energy in the cell. They contain the enzymes for the Krebs cycle, carry out oxidative phosphorylation, and are involved in fatty acid biosynthesis (Russell, 1992). Chloroplasts are defined by the same author as cellular organelles found in green plants, in charge of the photosynthetic process.

Organelle Genome Organization

According to Warren and Wickner (1996), "every membrane-bordered organelle in a particular cell type has a characteristic copy number, size, and position, which reflects its cellular function" (p.395). Mitochondria, for example, are often located near organelles that consume

energy, and their number is determined by the energy needs. For example, in mammals, they are numerous in muscle cells and are tightly wrapped around the flagellum. The cytoplasmic DNA forms DNA-protein complexes, called nucleoids (organelle nuclei), that can be seen clearly as bright dots under epifluorescence microscopy by staining with DAPI (4'6-diamidino-2-phenylindole) (Sodmergen et al., 1994).

Mitochondrial genomes of higher plants are unusually large, when compared with the animal mitochondrial genome (usually around 16 kb). Values in the range of 200 to 2400 kb were indicated by Ecke et al. (1989). This complex organization was first described for *Brassica campestris,* by Palmer and Shields (1984), mentioned by Ecke et al. (1989), as a 218 kb master chromosome, with one pair of a 2 kb direct repeat, that can be resolved into two subgenomic circles of 135 and 83 kb by recombination within this repeat.

Other plant species have more than one set of repeats. The multicircular organization of the normal maize mitochondrial genome presents a master circle of 570 kb with six pairs of repeats. Many possible subgenomic molecules can arise, due to recombination between direct repeats. The cytoplasmic male sterile (cms) maize mitochondrial genome is

even more complex, since six permutations of the master circle alone are possible (Gillham, 1994).

According to Ecke et al. (1989), the reason for this multipartite organization and high frequency of recombination remains unknown. Also, they mentioned that this kind of mitochondrial organization is not always the rule, as the *Brassica hirta* mitochondrial genome consists of a 208 kb master chromosome only, with no subgenomic circles.

However, despite their large size, plant mitochondrial genomes do not contain many more genes encoding proteins. The majority of the mitochondrial proteins are encoded in the nuclear genome (Gillham, 1994).

Birky (1994) indicated that only a minority of plant mitochondrial DNA (mtDNA) molecules are really circular in vivo. According to Bendich and Smith (1990), cited by Birky (1994), the maxicircle (or "genomic chromosome") could be a long linear concatamer of minicircles (or "subgenomic circles") that replicates as a rolling circle. However, since the minicircles do not contain the complete mitochondrial genome, every daughter cell must receive at least one mastercircle.

According to Stoneking (1996), one of the properties that makes mtDNA very useful for evolutionary studies is that it is present in multiple copies per cell. There are

several mtDNA genomes per mitochondrion, several hundred mitochondria per cell, and several thousand billion cells per individual. This author suggested that this makes us a population of mtDNA genomes.

According to Gillham (1994), the chloroplast genomes of a few land plants (mostly angiosperms) are very well characterized. The plant chloroplast genome is defined as one circular chromosome, with a very conserved DNA sequence. In striking contrast between the mitochondrial genomes of liverwort (*Marchantia polymorpha*) and angiosperms, their chloroplast genomes are very similar in organization and gene content. According to this author, the chloroplast genomes possesses two unique sequence regions and two large inverted repeats (typically 10-30 kb), always positioned asymmetrically, splitting the chloroplast genome into unique small and a large sequence regions.

Nuclear genes are organized in chromosomes, which are replicated once and each daughter cell receives one copy at cell division. In contrast, organelle chromosomes are chosen for replication and partitioned at random to daughter cells during cell division. According to Birky (1994), there is no loss of genes, because, in general, each organelle chromosome contains a complete genome (a complete set of mitochondrial or chloroplast genes). Organelle genes obey non-Mendelian laws: vegetative segregation (alleles of

organelle genes segregate during meiosis and mitosis), uniparental inheritance and intracellular selection (some organelle chromosomes may replicate faster than others, in the same cell, depending on their genotype). This author called the organelle genome the "relaxed genome." Birky (1994) also indicated that mutant alleles can be fixed by intracellular selection.

Organelle Division, Distribution and Inheritance

Kuroiwa and Uchida (1996) suggested that organelles in higher organisms might divide with the use of prokaryote-like "division rings" that we cannot yet visualize. They believe that "microbodies" are in control of organelle division, at least in certain Eukaryotes. Microbodies are common and consist of at least two types: glyoxysomes and peroxisomes, both of which contain the enzyme catalase. When studying the *Chlamydomonas merolae* organelle division, these authors observed the initial signs of a mitochondrial division(MD)-ring formation, with the movement of a microbody to the region between the mitochondrion and the cell nucleus. An MD-ring that is 40 nm wide and 40 nm thick forms in the cytoplasm at the point where the microbody has attached to the mitochondrion. These authors also pointed out that the separation of organelles following their

divisions to daughter cells is important for cytoplasmic inheritance.

Berger and Yaffe (1996) indicated that mitochondria will arise only from pre-existing mitochondria, in a coordinated expression of nuclear and mitochondrial genes. There is a role for the cytoskeleton in the positioning and movement of the mitochondria. This was observed when chemical agents that perturbed the cytoskeletal network also altered the mitochondrial distribution. According to these authors, the microtubules largely co-align with the mitochondria during *Saccharomyces pombe* cell division and have a central role in the mitochondrial distribution. However, the authors pointed out that there are differences in the mitochondrial behavior between the fission and budding yeast (*Saccharomyces cerevisae*). These authors also mentioned that a mitochondrion may not necessarily behave as a single, unitary structure, and that different components (e.g. matrix and the inner membrane) may possess different motility properties. These authors described two classes of proteins that facilitate the movement and the inheritance of mitochondria in yeast: cytoskeletal proteins and mitochondrial proteins. Two general mechanisms are probably involved: one has to do with "molecular motors" that bind the mitochondrial surface and pull the mitochondria along the cytoskeletal tracks. In the other mechanism, the

mitochondria would move via internal changes in morphology and the sequential binding and release of surface structures along a cytoskeletal scaffold.

According to Mogensen (1996), the polarization of chloroplasts during cell division may be mediated by microtubules, actin filaments and/or a biochemical gradient. However, this author indicated that more studies are necessary in this area.

Types of Organelle Inheritance

The study of organelle genetics began over 80 years, when Correns and Baur discovered in 1900 that mutations affecting the chloroplast phenotypes of higher plants frequently exhibit non-Mendelian inheritance (Kumar and Cocking, 1987). Interest in organelle inheritance was aroused only 10 years later, after the rediscovery of Mendel's laws. The study of organelle inheritance began with the use of phenotypic markers (mainly chlorophyll deficiency) and has more recently been extended by cytological and molecular approaches (Reboud and Zeyl, 1994).

Kuroiwa and Uchida (1996) mentioned that, in primitive cytoplasmic inheritance associated with sexual reproduction, male and female derived organelles are mixed in the zygote, and in some cases fused, permitting recombination of their

plastid DNA. They also mentioned that recombination of mtDNA in the slime mold *Physarum polycephalum* depends on mitochondrial plasmids and thus is analogous to bacterial sex (conjugation). According to these authors, in more advanced forms of cytoplasmic inheritance, uniparental transmission of organelles is generally seen.

The inheritance of organelles has been categorized into three types: maternal, paternal and biparental. The most common mode of organelle inheritance in angiosperms is uniparental-maternal. About one third of all higher plant genera at least occasionally inherit plastids biparentally (Mogensen, 1996). Most of these results have been obtained from studies with chlorophyll-deficient mutants, as chlorophyll markers (Smith,1989). Biparental, or even predominantly paternal transmission of chloroplasts has been documented in a number of angiosperms such as *Nicotiana tabacum* (Medgyesy et al., 1986; Yu et al., 1994), *Medicago sativa* (Lee et al., 1988), *Oenothera* (Chiu and Sears, 1993), *Coreopsis grandiflora* (Mason et al., 1994), *Turnera ulmifolia* (Shore et al., 1994), *Lens culinaris* (Rajora and Malhon, 1995), *Pelargonium* (Guo, 1995) and *Actinidia* (Cipriani et al., 1995).

Examples of paternal inheritance of mitochondria in gymnosperms are common and have been reviewed by Reboud and Zeyl (1994): in *Sequoia sempervirens* (Neale et al., 1989),

in *Pinus banksiana x P.contorta* (Wagner et al, 1991) and *Calocedrus decurrens* (Neale et al., 1991).

In contrast, there are few reports of deviations from maternal inheritance of mitochondria in angiosperms. This may be the result of either inherent differences between these organelles or the lack of mitochondrial phenotypic markers (Rebould and Zeyl, 1994). Erickson and Kemble (1990) reported that the paternal transmission of the mitochondrially associated plasmid can occur in *Brassica napus*. Fairbanks et al. (1988) observed that F1 progenies of alfalfa (*Medicago sativa*) inherited several large RNA molecules from both maternal and paternal parents. More recently, a predominant paternal transmission of the mitochondrial genome in cucumber (*Cucumis sativus*), was documented by Havey (1997).

Biparental inheritance of chloroplasts and mitochondria has been observed in intergeneric crosses involving *Hordeum* and *Secale* (Soliman et al. 1987) and *Festuca pratensis* and *Lolium perenne* (Kiang et al. 1994). Maternal inheritance of chloroplasts and paternal inheritance of mitochondria were observed in bananas (*Musa acuminata*) by Faure et al. (1994).

Examples of biparental inheritance of mitochondria in animals have been described for the mussel *Mytilus edulis*

(Hoeh et al., 1991) and the bark weevil (*Pissodes* sp.). An interesting point was raised with the work of Kaneda et al. (1995), where paternal mtDNA was detected in crosses between two different species of mice, but not in intraspecific crosses. The authors presented a model where the interaction between sperm mitochondria and components in the egg cytoplasm involves a receptor-ligand system. According to the model, receptor and ligand are mismatched in interspecific hybrids because of the evolutionary divergence, thus preventing efficient recognition and elimination of the sperm mitochondria. Hoeh et al. (1991) also agreed that "biparental inheritance of mtDNA may be a hybrid phenomenon" (p. 1489).

Heteroplasmy

Heteroplasmy can be defined as the presence of different types of mtDNAs coexisting within a cell or individual. Transplanting germ plasma into an egg carrying a different type of mtDNA should generate germ cells possessing two types of mtDNA in the cytoplasm, provided mitochondria in the donor germ plasma survive and are successfully transmitted to the next generation. Matsuura et al.(1989) induced mtDNA heteroplasmy by intra- and interspecific transplantation of germ plasma in *Drosophila*. They demonstrated that the heteroplasmic state persists in

female germ cells at least from heteroplasmic G_0 fly to the G_2 progeny. The authors concluded that since no significant decrease or elimination of donor mtDNA was observed and the heteroplasmic state was obviously retained in germ cells, it is clear that rapid purification of mitochondria with respect to mtDNA type does not occur, at least in early generations.

Organelle heterosis is defined by Smith (1989) as the occurrence of a heterotic response when organelles from different genotypes are mixed, either in vitro or in vivo, in a cytoplasmic hybrid. The author mentioned the work of Sarkissian and Srivastava (1967) who observed enhanced oxidative phosphorylation efficiency in artificially mixed mitochondria of particular maize inbreds when compared with the mitochondria of inbreds alone. Organelle heterosis/complementation requires that regular biparental inheritance of organelles occurs, although this was not always stated. According to Smith (1989), regular biparental inheritance of organelles could represent a method for achieving cytoplasmic hybridization.

Laipis et al. (1988) and Koehler et al. (1991) reported that nucleotide sequence differences can arise within a few generations in the mtDNA of Holstein cows. The authors implied that heteroplasmy can be considered a source of evolution of the mitochondrial genome. Koehler et al. (1991)

observed the replacement of Holstein cows leukocyte mtDNA by a sequence variant in just one generation. This implies, according to the authors, the existence of heteroplasmic cells in the cell lineage between the germ line of the mother and leukocytes of the progeny, and that the heteroplasmic state is transient relative to the total number of cells in this cell lineage.

Lee and Taylor (1993) detected a complete mitochondrial replacement during mating in the filamentous fungus *Neurospora tetrasperma*. In only three days following hyphal fusion, the nuclear acceptor strain mtDNA replaced donor mtDNA throughout the entire colony. They raise the point that perhaps both mitochondrial types have existed for short periods of time or in unequal stoichiometries and have gone undetected by their methods. These authors indicated that replacement can be due to the migration of one mitochondrion throughout the recipient mycelium coupled with destruction of the other type, to an organelle replication advantage of one of the types, or even to recombination between mitochondria, with the identifying features of one genome always being incorporated in the recipient. The possibility that variant mitochondrial genomes can be fixed in one generation should be considered in analyses of evolutionary relationships based on divergence of mitochondrial sequences (Koehler et al., 1991).

Kondo et al. (1990) found heteroplasmy due to an incomplete maternal transmission of mtDNA in *Drosophila*. This group used radioactively labeled probes that were specific to paternal mtDNAs. This method could detect as little as 0.03% paternal mtDNA when present in a sample. MtDNA heteroplasmy was also observed in *Drosophila mauritiana* by Solignac et al. (1983). Petri et al. (1996), found an extraordinary level of mtDNA sequence heteroplasmy within a species of European bat, and there was as much variation within an individual bat as there was within the entire population. These authors provided evidence to suggest that such heteroplasmy also exists in humans, although in reduced levels, compared to the bats.

Jazin et al. (1996) estimated the spectrum of mtDNA sequence heteroplasmy in the brain of normal individuals in order to determine the importance of heteroplasmy to aging and neurodegenerative disease. The level of heteroplasmy was assessed in five regions of the mtDNA in human brain by denaturing gradient gel eletrophoresis (DGGE). Analyses revealed high levels of heteroplasmy in the D-loop (a triple-stranded displacement loop, with no coding function), while no variability was detected in the coding regions. These authors observed a higher frequency of mitochondrial heteroplasmy in the older individuals (about 90 years old) in comparison with a 28 year old individual. Small

insertions and deletions increased 7.7-fold, but point mutations did not show any significant increase with the aging process. This group indicated that further experiments measuring the accumulated damage in the coding region will be needed to clarify the functional importance of heteroplasmic mutations in aging human brain.

Turner et al. (1995) studied the inheritance of kinetoplast DNA in *Tripanosoma brucei* (a protist). The kinetoplast is a unitary organelle that replicates and segregates into each daughter cell at mitosis. It is found in the matrix of the cell's single, giant mitochondrion (Gillham, 1994). It contains maxi and minicircular DNA molecules and the maxicircles are equivalent to mtDNA, except they appear not to contain tRNA genes. Minicircles encode the vast majority of guide RNAs involved in the mitochondrial RNA editing. In the progeny, maxicircles are inherited uniparentally, and minicircles are inherited biparentally. However, Turner et al.(1995) demonstrated that the inheritance of maxicircles is biparental in some clones during the early growth stages of hybrid progeny resulting from a genetic cross. Subcloning and further vegetative expansion showed that the mixtures of maxicircle genotype were unstable and quickly became fixed. They pointed out that 140 generations (35 days) would be necessary to achieve

stability. In contrast, there is no requirement for the minicircles to segregate with strict fidelity.

In contrast to mitochondria, Smith (1989) and Chiu and Sears (1988) in studies using species that inherited the plastids biparentally, did not observe evidence of chloroplast recombination in a population of sexual hybrids.

Uniparental x Biparental

The inheritance strategy adopted by a particular organelle can vary among cell types and organisms (Warren and Wickner, 1996). Even with a particular cell type, different organelles could use different strategies. The inheritance of cellular organelles, therefore, differs fundamentally from the inheritance of chromosomes, where a single, universal strategy is used, based on the mitotic spindle. Reboud and Zeyl (1994) also suggested that uniparental and biparental inheritance are not fixed alternatives. According to these authors, they are reversible conditions, whose frequencies in the population can respond to selection. These authors also raised two hypotheses that can give rise to opposite expectations: (i)If only the nuclear genome is responsible for organelle inheritance, selection for such control should be relaxed in selfing angiosperms, where a potential conflict between organelles of different origins is much less frequent.

Biparental inheritance should therefore be more common in selfing than in outcrossing species; (ii)But, if organelles can drive their own inheritance, a biparentally inherited plastid will invade an outcrossing population, while in selfing species such plastids would only replace other copies of themselves. Thus, according to this second hypothesis, biparental inheritance should be more common in outcrossing than in selfing species. After reviewing different studies analyzing organelle inheritance in different plants, these authors concluded that these data support the hypothesis that plastids can influence their inheritance. For example, paternal and biparental inheritance of plastids is found in all gymnosperms, which are known to be outcrossers.

The probability of failing to recognize occasional paternal transmission is usually high. The number of hybrid plants, according to Smith (1989), typically subjected to organelle inheritance analysis has usually been quite small. Also, the organelle extraction, DNA isolation and visualization procedures used in most of the organelle studies generally are not sensitive enough to detect small amounts of paternal DNA (stoichiometrically underrepresented in hybrid plants) should it be present. Milligan (1992) and Avise (1991) also mentioned that unless very low probabilities of paternal or biparental

inheritance can be detected, biologists may incorrectly assume strict maternal inheritance. Milligan (1992) used a binomial approach to show that a progeny may contain paternally inherited chloroplasts with a probability of P=0.0001-0.025. Extremely large sample sizes would be required to distinguish between uniparental and biparental organelle inheritance. This distinction is very important, according to Milligan (1992), when considering the population genetics of organelles. The possibility of a paternal transmission opens the possibility of recombination among distinct organelle genomes, and this can alter the patterns of diversity observed in organelle genomes.

Erickson and Kemble (1993) also suspected that there may be varying degrees of paternal or biparental inheritance of mitochondria in plant species but that studies to detect it have been too few and inadequate. These authors used cytoplasmic male sterility as a marker to prove that paternal mitochondria can be transmitted to the progeny of a sexual cross in rapeseed (*Brassica napus*). They were able to transfer paternal mitochondria to the progeny in only one of the four crosses. An average of 1 in 15 progeny of this cross contained the paternal mitochondria. Also, Avise (1991) suggested that a paternal mtDNA might rarely, but quickly, colonize and dominate a

maternal line. Under a neutral model, according to this author, the expected frequency of such takeovers is simply the ratio of sperm:egg mtDNA numbers in a zygote, which is probably less than 1:2000 in most animal species.

Erickson and Kemble (1993) suggested that the female genotype has an influence on whether paternal mitochondria will survive and replicate in the progeny of a sexual cross. But, the authors also indicated that they cannot determine if the genetic factors responsible for pollen transmission of mitochondria on the female side are cytoplasmic, nuclear or both. The authors concluded that genes active during pollen development and following fertilization are responsible for the maternal inheritance of mitochondria in higher plants.

Erickson and Kemble (1993) reported the effect of ploidy level on the transmission rate of paternal chloroplasts through the pollen. The authors mentioned the work of Massoud at al. (1990) where tetraploid alfalfa plants transmitted plastids to their progeny at a higher rate than diploid genotypes, regardless of whether they were males or females in a cross.

Environmental conditions also play a role in the male cytoplasmic inheritance, according to Yu and Russell (1994). They studied the sperm-cell organization of *Nicotiana tabacum* under different environmental conditions.

Sperm cells were serially reconstructed to evaluate their quantitative cytological organization and that of their organelles. They observed that the size of the nucleus and the number of mitochondria were larger in flowers grown in growth chambers under warmed controlled conditions, whereas the number of plastids was the same or maybe slightly higher in flowers under cooler greenhouse conditions. The authors pointed out that further research would be necessary to verify if this kind of environmental influence occurs in other flowering plants.

Mechanisms Involved in Organelle Inheritance

Vaughn (1981) described two contrasting theories to explain organelle inheritance in higher plants: 1) physical exclusion of the organelles from the generative cell and 2) organelle alteration leaving debilitated organelles that would be incapable of genetic transmission, even if included in the generative cell. This author investigated the ultrastructure of pollen grains of a severely debilitated plastome mutant of *Pelargonium* that is not transmitted via pollen. Usually, this is one of the angiosperms presenting paternal inheritance of chloroplasts. Because the plastids are not physically excluded from the generative cell, although they are not transmitted via pollen, the theory of physical exclusion is

inadequate to explain the chloroplast inheritance in this mutant. Debilitated organelle genomes are not transmitted to the progeny with success despite the inclusion of these organelles in the generative cell. According to Vaughn (1981), this conclusion is consistent with the organelle alteration hypothesis for the control of organelle inheritance. The same author described myelin-like figures, that are no longer recognizable as mitochondria, as possible degenerate mitochondria. Similar "unknown bodies" were found by other authors (Clauhs and Grun, 1977, and Hagemann, 1979, mentioned by Vaughn, 1981) and were assumed to be degenerate mitochondria or plastids. Hause (1986) also observed myelin-like structures, besides intact plastids and mitochondria. The author pointed out that by the first pollen mitosis during the pollen development of *Pisum sativum,* the number of mitochondria appears to be 5-10 times higher than that of plastids. By this time, mitochondria and plastids are randomly distributed within the generative and vegetative cells. The author mentioned that the mitochondria of the vegetative cell contain a higher number of cristae and they are bigger in diameter than the generative cell mitochondria.

The organelle exclusion hypothesis, first put forward by Sager (1972), is supported by Ikehara et al. (1996). When studying the maternal chloroplast inheritance in

Chlamydomonas reinhardtii, these authors noticed that in the zygotes, the chloroplasts from the male parent (mating type minus, mt^-) disappear within 40-50 minutes after mating, while those from the female parent (mating type plus, mt^+) persist. Thus, the preferential digestion of chloroplast nucleoids was suggested as the main reason for the maternal inheritance of the plastid genes. According to these authors, soon after the zygote formation, specific mRNAs are synthesized in the cell nucleus of mt^+ cells, which code for proteins that directly or indirectly activate a calcium dependent nuclease to digest mt^- nuleoids in the zygotes. They mentioned the nuclear gene *zys1B* as tightly related to preferential digestion of male origin chloroplast nucleoids. The Zys1B protein was detected in early stages of the zygote formation, then decreased to an undetectable level with the completion of the preferential digestion. The turnover of the transcript is rapid and a putative amino acid sequence of this protein has cysteine and glutamine rich domain, indicating that it could be a transcription regulator.

Ikehara et al. (1996) also investigated whether higher number and higher DNA amount (fivefold) of chloroplast nucleoids could disturb this process of preferential digestion and maternal inheritance. Their results showed that this preferential digestion occurred with the appearance of the Zys1B protein, although a slightly longer

period was needed to complete the digestion of chloroplast nucleoids in large number.

In *Chlamydomonas*, according to Nakamura et al. (1992), endo-exonucleolytic activity of Nuclease C is high enough to completely digest about 10 chloroplast nucleoids, dispersed in the male chloroplast, in just 10 min. In their research, when this enzyme was added externally to the cells whose cell wall, membrane and chloroplast envelope have been biochemically punctured, it specifically digested male chloroplast nucleoids, rather than those of female. According to these authors, plants like *Rhododendron kaempfer, Zygocactus truncatus, Oenothera lacinata* and *O. speciosa,* showing biparental chloroplast inheritance, probably have a low activity nuclease C, allowing the intact paternal chloroplast nucleoids, or plastid DNA to remain. Nucleases are also referred by Lee and Taylor (1993) and Kuroiwa and Uchida (1996), as possible destruction agents of one of the parent's organelle genome. They also mentioned that no mechanism of methylation seems to be involved in the process of organelle inheritance.

Lee and Taylor (1993) referred to the theory of exclusion as the heterokaryon incompatibility associated with the mating type alleles, which immobilizes the cytoplasm of the male. Migration of nuclei into one partner without concomitant mitochondrial migration has been

reported by these authors in a work with the filamentous fungus *Neurospora tetrasperma*. These authors mentioned a possible extrinsic feature, maybe associated with nuclear acceptance or female behavior, which confers a replication advantage or destructive ability on the maternal mtDNA.

Mogensen (1988) described several mechanisms that have been considered responsible for the uniparental inheritance of organelles, in even more detail: "(i)exclusion of the organelles from the generative cell or one sperm cell during the first or second pollen mitosis, respectively; (ii)deletions in plastid DNA, degeneration of organelles, or elimination of organelle-containing cytoplasm during microspore, generative, or sperm cell maturation; (iii)organelle exclusion during gamete fusion; (iv) degeneration, lack of replication, or compartmentalization into suspensor cells after zygote formation" (p. 2594). This author indicated that structural evidence for the exclusion of male cytoplasm has been reported for spinach and cotton, in which two membrane-bound, enucleate cytoplasmic bodies were found within the degenerated synergid shortly after fertilization. Mogensen's (1988) hypothesis has to do with the relocation of the sperm nucleus once an opening in the egg is created, which would result in the sperm cytoplasm being left behind within its original plasma membrane. Subsequent closing of the opening

at the point of fusion could occur by a process of membrane reformation, involving vesicles present within the egg and sperm cell or by a membrane constriction process. The author pointed out that this mechanism may be important during sexual reproduction for the nontransmission of genetically altered male organellar genomes, such as large deletions in the chloroplast DNA, known to occur in microspores of wheat and barley.

Hause (1986) indicated that Hagemann (1983) summarized three modes of plastid behavior that lead to uniparental inheritance: (i) *Lycopersicum*-type, where an extreme unequal distribution of plastids during the first pollen mitosis causes the lack of these organelles in the generative cell; (ii) *Solanum*-type, when after the first pollen mitosis some plastids are present in the generative cell. They degenerate and are not present in the mature sperm cells. (iii) *Triticum*-type, when intact plastids are transmitted into the sperm cells, but during the fertilization only the sperm nucleus enters the egg cell. The cytoplasm of the sperm cell remains outside the egg.

Reboud and Zeyl (1994) affirmed that during gametogenesis, organelle loss results primarily from the formation of "cytoplasmic projections" that are subsequently discarded from the sperm cell body. These authors also pointed out that no exclusion mechanism is

completely effective on its own. Probably they operate in sequence, during both gametogenesis and embryogenesis. There must be a trend, where the more evolved is the organism, more numerous are the mechanisms employed.

Kuroiwa and Uchida (1996) noted a huge amplification of organelle DNA in egg cells during *Pelargonium zonale* oogenesis, which accompanies an increase in the egg cell volume. This phenomenon complements processes such as unequal division of first pollen mitosis and preferential digestion of organelle DNA in generative cells to yield a complete uniparental inheritance of organelle genomes.

Hause (1986) mentioned the theory of Russel and Cass (1983) concerning the biparental inheritance of plastids in *Plumbago zeylanica*. During the prophase of the second pollen mitosis, they observed a polarization of plastids in the generative cell. In consequence, one sperm cell contains all plastids and a small amount of mitochondria; in the other sperm cell, there was the majority of mitochondria and no plastids. During the fertilization process, only the sperm cell containing the plastids fuses with the egg cell, and causes biparental inheritance of plastids in this species. The other sperm cell, according to Reboud and Zeyl (1994), will fuse with the central cell, which will become the endosperm. The number of paternal mitochondria to the endosperm is greater than that

transmitted into the egg as the result of preferential fertilization by the mitochondrion-rich dimorphic sperm cell. In an evolutionary paradox: mitochondria behave suicidally by entering a sperm cell which will never transmit them, although this probably increases the fitness of the embryo (Reboud and Zeyl, 1994). According to these authors, in such cases, mitochondrial segregation must be controlled by nuclear genes. Paternally-derived nuclear genes in the embryo probably benefit from the presence of mitochondria in the endosperm, perhaps increasing the allocation of material resources to the embryo.

Why Uniparental Inheritance?

Hastings (1992) postulated that isogamy (where all gametes are identical in size and function) is disadvantageous as it allows deleterious cytoplasmic organisms (such as intracellular parasites) to spread through the population when cytoplasms are shared at fertilization. Conversely, according do this author, the uniparental inheritance restricts deleterious genomes and they cannot spread through the population. He even argued that once uniparental inheritance is established, the "male" gametes which contribute no cytoplasm may become small and anisogamy may evolve. Therefore, according to Hastings (1992), uniparental inheritance of cytoplasm arose

in response to the presence of deleterious cytoplasmic agents.

One example of a deleterious genome, mentioned by this author, would be the so-called "selfish mitochondria," which increases its own rate of replication, at the cost of reduced metabolic activity. The overall frequency of selfish mitochondria is a function of two processes: their initial frequency rises due to their replicative advantage, but eventually falls, as cytoplasms without selfish mitochondria dominate the population due to metabolic advantage. The number of mitotic divisions would be determinant in the ability of the selfish mitochondria to be transmitted. A cytotype initially containing a mixture of "selfish" and wild type mitochondria will eventually, after enough mitotic divisions, produce cytoplasms containing exclusively wild type or "selfish mitochondria." This phenomenon was described by Hastings (1992) as Mitotic Segregation. This author indicated that mutations producing "selfish mitochondria" are likely to arise continuously, and their gradual accumulation in the population may result in a continuous decline in mean fitness.

Reboud and Zeyl (1994) suggested that monogametic transmission and selective silencing may have evolved to avoid organelle recombination. According to these authors, uniparental inheritance may prevent the invasion by

transposons, which seem to be less abundant in chloroplast genomes than in the mitochondria and nuclei.

Hastings (1992) indicated that, in extant species, it appears that a small amount of paternal "leakage" may occur. This would be a transitory phenomenon, as natural selection favors nuclear alleles which reduce this leakage. The amount of leakage observed in extant species represents the balance between the benefit of decreased leakage which inhibits the spread of deleterious cytoplasmic agents and the advantages of increased leakage which aids the incorporation of advantageous mutations across the lineages.

Allen (1996) indicated that the respiratory electron transport and ATP synthesis in mitochondria are accompanied by the generation of mutagenic free radicals of oxygen, causing damage to the mitochondrial genetic system. The author indicated that mtDNA suffers oxidative damage at about ten times the rate of nuclear DNA. There is a positive feedback loop: free radical mediated mutagenesis of mtDNA may initiate and promote further mutagenesis through its effects on the structure and function of respiratory chain proteins, and on mitochondrial gene expression. The author pointed out that there is an inverse correlation between life span and metabolic rate, and that in plants, chloroplast genomes may be subject to a similar cycle of

redox damage from electron transport in photosynthesis. Allen (1996) even indicated that the proximity of certain regions of plastid and mtDNA to the membrane-bound photosynthetic and respiratory electron transport chains should be expected to lead to mutation "hot spots" (regions of DNA close to sites of O_2 generation). Allen (1996) also described that in animals, a similar positive feedback loop ("vicious circle" of energy loss) has been proposed as an explanation of aging, whereby mitochondrial division is impaired and becomes incapable of replenishment in postmitotic cells such as those of muscle.

However, the offspring do not inherit their parents' somatic degeneration. The author suggested that the positive feedback loop is broken in mitochondria of the female germ line, from which all the mitochondria derive (escape of female germ line from mitochondrial ageing). In a very interesting hypothesis, Allen (1996) indicated that the mitochondria of the female germ line have a repressed bioenergetic function, avoiding mutagenesis from products of respiratory electron transport. In consequence, their genomes would survive and replicate with minimal damage. Allen (1996) named "Promitochondria" as the mitochondria of the male germ line and of somatic cells of both sexes: bioenergetically functional but genetically disabled. This would lead to the elimination of damaged, paternal mtDNA at

or before fertilization. The promitochondria persists in plants in meristematic cells, prior to differentiation of somatic and germ cells. The author suggested that the size, motility and number of gametes can be secondary characteristics to define male and female. According to his hypothesis, oxidative phosphorylation is the difference between sexes.

Specific predictions arise due to Allen's (1996) hypothesis: (i)female germ-line mitochondria have a repressed bioenergetic function; (ii)cells of the female germ line are sequestered from somatic cell lines at an early stage in development, prior to differentiation of promitochondria (not true for plants); (iii)female gametes are relatively long-lived, having the lowest metabolic rate consistent with viability, and usually are few in number; (iv)mitochondrial damage may eventually accumulate and natural selection would favor the elimination of individual females that are still capable of passing mutant mitochondria. This would have to do with the limit of the female reproductive phase; (v) female gametes depend on import of ATP from somatic cells for energy; (vi) the male germ-line mitochondria is a genetic dead end, committed to short-term energy production. So male gametes would be short lived, produced at any stage of a lifetime.

Hastings (1992) mentioned that the spread of deleterious elements, as "selfish mitochondria," may constitute another action pressure favoring the transfer of genes from mitochondrial to nuclear DNA. Allen (1996) proposed that the major reason for retention of certain genes in the organelles is a requirement for redox control of plastid and mitochondrial gene expression. This minimizes free radical damage to the cell as a whole. "Eukaryotes and their nuclear genes owe a debt to the altruistic mitochondria and chloroplast, which occupy the eukaryotic cell's most hostile internal compartments" (p. 139). Avise (1991), referred to the endosymbiont origin of the organelles, when the author postulated that "the symbiont ensures its own survival by keeping its fingers on the jugular vein of cellular energy flow" (p. 55).

The Nuclear Influence

The majority of components necessary for mitochondrial and chloroplast functions are supplied by genes encoded in the nucleus. The nucleus is involved in mtDNA replication, recombination, and/or mitochondrial segregation (Mackenzie et al., 1994). One example of nuclear gene regulation of mitochondrial function is the existence of a single nuclear gene (*Fr*) able to restore the fertility of CMS (cytoplasmic

male-sterility) in common bean, by altering the mitochondrial genome (Mackenzie and Chase, 1990).

Another example of nuclear genome influencing the mitochondrial genome organization was described by Sakamoto et al. (1996) with a (maternal inherited) distorted leaf mutant of *Arabidopsis,* induced by the recessive nuclear mutation: *chloroplast mutator(chm)*. This mutation causes the preferential amplification of substochiometric mtDNA configurations (present in very low amounts). *CHM* induces mutations not only in mtDNA, but also changes in chloroplast morphology and function. However, the authors pointed out that it is difficult to correlate the morphological change with chloroplast mutations, because mitochondrial mutations can affect chloroplast structure and function. They propose that this nuclear gene in *Arabidopsis* has a function similar to *Fr* in common bean. The function of CHM and other nuclear products affecting mitochondrial genome organization is still to be elucidated. According to Sakamoto et al (1996), it is possible that these proteins interact with a specific region of the mitochondrial genome and preferentially maintain master molecules, maybe securing their distribution along with the mitochondrial division.

The fact that Gymnosperm plants transmit mtDNA maternally, but chloroplast DNA paternally, suggests some active regulation of organelle transmission, since both

genomes must be subject to the same physical constraints imposed by the relative sizes of the egg and pollen cytoplasms (Avise, 1991). Tilney-Bassett (1994) has shown that the genetic transmission of plastids is under nuclear control. The relationship between nuclear and chloroplast genomes was discussed by Smith (1989) when describing a special type of chlorophyll deficiency, termed "hybrid variegation" that appears to result from an incompatibility of some sort between the nuclear and cytoplasmic genomes of the parents involved in a cross.

Gotoh et al. (1995) described nuclear-cytoplasmic interactions and sometimes lethal recombination of genes, causing reproductive incompatibility between two populations of the spider mite, *Tetranichus quercivorus*. They mentioned that the cytoplasmic factor responsible for the incompatibility in this species may be cytoplasmic heritable agents such as mitochondria. These authors indicated that examples of incompatibility between the cytoplasmic factors of one population and the nuclear genes of another population are known in insects, plants such as wheat, and in cybrid plants possessing the *Atropa* genome and the *Nicotiana* plastome.

Another example of nuclear influence over the mitochondrial genome has to do with RNA editing. This process results in mitochondrial RNA alterations, which may

change the meaning of the genetic information. Most are C-U transitions that occur in open reading frames, but a few are observed in intron sequences (Pring et al., 1993 and Wilson and Hanson, 1996). According to these authors, the specificity of RNA editing in plant mitochondria is probably determined by nuclear gene(s), according to site affinity and accessibility.

Small et al. (1987) observed in maize changes in stoichiometry of different molecules in response to different nuclear backgrounds. They suggested that an explanation for sudden genomic reorganization of the mitochondrial genome is selective amplification of pre-existing sub-stoichiometric (possibly undetectable) molecules (perhaps together with a reduction of previously abundant molecules).

According to Smith (1989), in the zygote of at least some species, a signal is produced possibly by nuclear gene(s), which determines whether maternal or paternal plastids or both will be propagated and in what proportions as the embryo develops.

Citrus

Most of the citrus scion and rootstock cultivars grown today are not a product of breeding programs, but originated by chance during seedling selections or bud mutations

(Grosser and Gmitter, 1990). Despite the immense genetic variability in the genus *Citrus* and related genera, the conventional breeding has had a limited role in terms of developing new varieties. According to these authors, this is due to several aspects of the citrus reproductive biology: heterozygosity and inbreeding depression, pollen and ovule sterility, sexual incompatibility, apomixis and nucellar polyembryony, and juvenility.

History of Somatic Hybridization

Regeneration from *Citrus* protoplasts began after Kochba et al. (1972), cited by Grosser and Gmitter (1990), reported the production of *Citrus sinensis* embryogenic callus from the nucellar tissue of cultured ovules. According to these authors, the first example of a successful somatic hybridization in *Citrus* was an intergeneric allotetraploid hybrid produced by Ohgawara et al. in 1985 between embryogenic protoplasts of *Citrus sinensis* 'Trovita' and *Poncirus trifoliata* leaf protoplasts. The production of a somatic hybrid plant between the sexually incompatible *C. sinensis* and *Severinia disticha* by Grosser et al. in 1988, demonstrated that protoplast fusion is a viable means of bypassing barriers to sexual hybridization. Other authors also reported their achievements in terms of somatic hybridization/cybridization including Ohgawara et al.

(1994), Vardi et al. (1987a, 1987b and 1990), Grosser et al. (1996) and Saito et al. (1994).

Methods Used for Somatic Hybridization

Protoplasts can be fused by chemical or electronic methods. Grosser and Gmitter have been using the polyethylene glycol (PEG) method since 1984. It is recommended because it is simple, efficient, inexpensive and does not seem to interfere with protoplast viability.

Protoplasts can be isolated from various sources including leaves, embryogenic callus, embryogenic suspension cultures, nonembryogenic callus, and flower bud tetrads (for haploid protoplasts) (Grosser and Gmitter, 1990). According to these authors, the requirement for plant regeneration following fusion has been that protoplasts from one of the parents must have embryogenic capacity. For general somatic hybridization, protoplasts from embryogenic callus or suspension cultures should be fused to a non-totipotent source of protoplasts such as those derived from leaves.

According to these authors, the somatic hybrid selection (the identification and separation of somatic hybrid plants from unfused parental material) is a key part in the whole process. Protoplasts of one parent are obtained from non-embryogenic tissue, reducing the possibility of a whole plant recovery from this parent. Grosser and Gmitter

(1990) cited the work of Ohgawara et al. (1985), where a high concentration of sucrose in the protoplast culture medium can inhibit the regeneration of the embryogenic parent. Together these processes prevent unfused protoplasts from undergoing somatic embryogenesis.

True somatic hybrids, according to Grosser and Gmitter (1990), have a vegetative morphology generally intermediate to the donor parents, tetraploid chromosome numbers, and what the authors called a composite expression of DNA or gene products markers. Among the techniques available for hybrid verification they described: mitotic chromosomes counting, visual evaluation, molecular characterization by gel eletrophoresis of DNA or isozymes, or even chromatographic separation of leaf oils. These authors pointed out that none of these methods is sufficient by itself; each is subject to limitations. The hybrid verification should include morphology evaluation, cytogenetic, and molecular characterization.

In contrast to somatic hybrids, "cybrids" contain the nucleus and exhibit the general morphology of the recipient parent, but contain the organelle genomes of the donor parent. Vardi et al. (1989) used mitochondrial probes for cybrid verification. These authors have been using the "donor-recipient" protoplast fusion method. The nuclear division of donor protoplasts is arrested by either X or

gamma irradiation, and the cytoplasm of recipient protoplasts is inactivated by an antimetabolite such as iodoacetate. Success in the "donor-recipient" method of cybridization depends on the ability of organelles to withstand the radiation that will disrupt the nuclear DNA. The differential effect of radiation on the nuclear and organellar DNA is probably due to the high number of organelles and organelle genomes present. Also, the mitochondrial structure may serve as a shield for the DNA, against the irradiation effects.

A different definition for cybrids was presented by Bonnema et al. (1992) and Kumar and Cocking (1987), where cybrids or cytoplasmic hybrids are defined as special cases of asymmetric somatic hybrids since they contain the nuclear genome of only one of the protoplast fusion partners and cytoplasmic organellar genomes of both parents.

According to Earle (1995), as the fusion product divides and produces callus or plants, the organelle populations segregate to give different, more or less stable combinations (Birky, 1978, reviewed by Earle, 1995). This process can be affected by the specific experimental conditions used during the creation of the fusion product.

Saito et al. (1993) observed that through the cell fusion between nucellar callus cells and mesophyll cells in two different citrus combinations, they obtained regenerated

plants resembling the mesophyll parent, in addition to the expected somatic hybrids. These authors investigated the mitochondrial genome composition of these plants. All the clones that resemble the mesophyll parents (and contain their diploid nuclear genome) have the nucellar callus parent mitochondrial genome. These plants are therefore cybrids. Considering the fact that the mesophyll parent does not have the regeneration ability, possibly the mitochondria of nucellar-derived cells play a significant role in *Citrus* embryogenesis. Grosser et al (1996) discussed the cybridization requirement for plant regeneration from citrus leaf protoplasts following somatic fusion. These authors mentioned that the origin of these cybrid cells could be from successful protoplast fusion accompanied by unsuccessful nuclear fusion and loss of the embryogenic parent nuclei, or by incomplete fusions where the protoplast of the embryogenic parent ruptured during the fusion process and resulted in only partial transference of its contents.

According to Smith (1989), one of the goals of organelle research is the transfer of a cytoplasmic trait from a given genotype into the nuclear background of a second genotype, often in cases where the two genotypes could not be hybridized sexually. Direct fusion-mediated transfer of mitochondrially encoded traits (like cms) to other lines selected as parents for hybrid production is an

attractive application for the somatic hybridization (Earle, 1995 and Akagi et al., 1995). Yamamoto and Kobayashi (1995) reported the production of a cybrid having the nuclear genome of *C. sinensis* and the cytoplasmic genome of *C. unshiu*, usually male sterile, a very desirable characteristic in terms of obtaining seedless fruits.

In a plant breeding program it would be quite valuable to achieve unilateral cytoplasm transfer relatively quickly leading to the exchange of cytoplasm without backcrossing. This is particularly important when we consider that methods for gene transfer into higher plant mitochondria are not yet available. Stable transformation of higher plant chloroplasts has been accomplished via particle bombardment (reviewed by Earle, 1995). However, this author observed that unlike backcrossing, only some of the plants recovered are likely to contain the introgressed cytoplasm, and these will probably differ in their mtDNA arrangements. Also, somaclonal variation and/or transfer of some unwanted nuclear DNA from the donor to the recipient in spite of irradiation pretreatment, may occur during the use of methods like the "donor-recipient" cybridization process.

Organelle Analysis in Somatic Hybrids

Methods of Organelle Analysis

Two general approaches for the analysis of mtDNA in putative somatic hybrids, are described by Earle (1995). First is the use of ethidium bromide stained gels of restriction enzyme-digested mtDNA. Efficient isolation of mtDNA may be a limitation in this approach. The second strategy is the hybridization analysis of total DNA isolated from the fusion products and parents. This permits the examination of small amounts of fusion-derived material (such as calli weighting as little as 50 mg) and multiple loci (by hybridizations with nuclear and organelle probes on a single DNA gel blot). The first step is to find combinations of restriction enzymes and probes that reveal clear differences between fusion parents. Usually the probes are cloned genes. However, gene-containing fragments, mtDNA regions from homologous and heterologous sources, cosmid clones carrying mtDNA, or simply total mtDNA from one fusion partner, also can be used.

Most studies of mitochondrial genomes after fusion, include only the types of analysis described above. However, a few studies (reviewed by Earle, 1995) provided more detailed information, such as mapping of the DNA regions contributed by each fusion parent (Morgan and Maliga, 1987; Honda and Hirai, 1992), sequencing of a recombinant

mitochondrial gene (Rothenberg and Hanson, 1988) and Northern analysis of transcription (Rothenberg and Hanson, 1988).

Organelle Genome Stability

Samolylov et al. (1996) indicated that in contrast to the array of recombinations that occur between the two nuclear genomes, the heteroplasmic state of somatic hybrid cells almost always sorts out quickly leading to uniparental transmission of chloroplast DNAs. By contrast, according to these authors, the mitochondrial genomes often undergo rearrangement and novel types of mtDNA in somatic hybrids are frequently observed. Motomura et al. (1996) observed mitochondrial recombination between parents in the product of an electrofusion between 'Seminole' tangelo and *Severinia buxifolia*. In contrast, no recombination was observed in the chloroplast genome of the products, and the sorting out of plastids was a rapid process.

Indeed, according to Earle (1995), mtDNA "rearrangement" or "non-parental mitochondrial genome" are really more appropriate terms to describe the "novel" mtDNA configurations, considering that differential replication of subgenomic molecules from the fusion partners, rather than intergenomic recombination, might be responsible for these observed unique fragments. This author indicated that,

because regeneration from fused protoplasts always involves a callus stage, mtDNA changes seen in somatic hybrids or cybrids might result from spontaneous culture-related changes, rather than to the fusion event per se.

Honda et al. (1991) mentioned by Earle (1995), worked with tobacco fusion products. They observed many different types of mtDNA rearrangements in small fusion-derived calli. However, when checking leaves from regenerated plants, a more uniform DNA was found.

Earle (1995) indicated that in the work of Morgan and Maliga (1987) *Brassica napus* cybrid calli and calli from unfused protoplasts both contained the same apparent modification in the mtDNA. Its appearance may be related to an intragenomic recombination *in vitro* or maybe is just an amplification of a band present at very low levels in the original plant (a "sublimon"). However, Nagy et al. (1983), reviewed by Earle (1995), observed mtDNA changes only in tobacco plants derived from fusions, not in plants regenerated directly from protoplast or callus culture of similar protoplasts.

Gleba et al. (1985), cited by Kumar and Cocking (1987), showed that heterozygosity for parental types is relatively stable in hybrid/cybrid plants and can be maintained even in their sexual progeny. According to the authors, this would

provide an unique opportunity for chloroplast DNA recombination between the two types of parental chloroplasts. They raise the point that usually the protocols fail to achieve this goal due to a lack of a stringent selection system, and also possibly due to a very low frequency of chloroplast genome recombination. According to Earle (1995), both inter- and intramolecular recombination appears to be quite common among mtDNAs in plants. In contrast, this author and Malone et al. (1992), mentioned that recombination of chloroplast DNA after fusion is very rare and there is only a single case of a putative chloroplast recombination in higher plants (Medgyesy et al., 1985).

Changes in mtDNA from regenerated plant to progeny, were also observed by Sakai and Imamura (reviewed by Earle, 1995) in *Brassica napus* cybrids obtained after fusion with irradiated cms radish line. Indeed, Earle (1995) pointed out that the mtDNA alterations that occur after fusion are not completely random. Rather, some regions look like "hot spots" for rearrangements. Rothenberg and Hanson (1988), reviewed by this author, identified some of these regions, and through sequencing and computer searchers, even provided some information about how the novel fragments were produced. They did extensive studies of a novel recombined *atp9* gene in progeny of a *Petunia* somatic hybrid obtained by

fusion of fertile and cms lines. By sequencing this gene, they showed that it contained the 5'end of one parental *atp9* gene (fertile *P. hybrida*) and the 3'end of an *atp9* gene from the other fusion partner (cms *P. parodii*). The coding region of the gene was identical in both parents and was not altered in the somatic hybrid. Portions of the 5' and 3' flanking regions were also the same in both parents. In the somatic hybrids, recombination apparently took place within a 414 bp region of homology between the two genes. Homologous recombination within the coding region of a mitochondrial gene was also detected in somatic hybrid calli of tobacco (Honda and Hirai, 1992). Rearrangements of mtDNA after fusion might facilitate wide hybridizations by achieving more favorable nuclear-cytoplasmic interactions through selection against mtDNA regions that did not interact well with the nucleus of the other fusion partner (Earle, 1995).

Morgan and Maliga (1987), cited by Earle (1995), examined small fusion-derived *Brassica* calli with one probe and concluded that mitochondrial segregation was complete in some (but not all) calli within 19-22 generations after fusion. More than half of these calli contained all regions from one partner plus some regions from the other, as well as some novel fragments. This result suggests a gradual loss of some regions independent of others, perhaps by

"independent segregation or differential replication of subgenomic mtDNA molecules" (p. 568). Kumar and Cocking (1987) pointed out that although the fusion products initially contained a mixed population of parental chloroplasts, somatic hybrid/cybrid plants subsequently recovered usually possess only one or the other parental chloroplast type. Furthermore, the authors mentioned that chloroplast sorting out appears not to be influenced by the degree of sexual compatibility of the fusion partners.

Earle (1995) mentioned that chloroplasts and mitochondria segregate independently after the somatic fusion and suggested that there is no strong tendency for the two types of organelles to move together except insofar as compatibility with the nuclear genome is limiting or strong selection for chloroplast traits is applied. The contrast between the behavior of plastids and mitochondria may be related, according to Kumar and Cocking (1987), to the structural organization at the membrane level of these cytoplasmic organelles, and also to differences in their genomic organization. DNA recombination is the rule governing plant mitochondrial genome organization.

Factors Influencing Organelle Inheritance

Samolylov et al. (1996) found no correlation between the nuclear genome composition and the transmission of

chloroplast and mtDNA during the production of asymmetric somatic hybrid plants between an interspecific tomato hybrid and eggplant. A different result was obtained by Bonnema et al. (1992). They decided to test if the inheritance of the organelle genomes is influenced by the nuclear background of the protoplast fusion product. Working with a collection of tomato symmetric and asymmetric hybrids, and cybrids between tomato (*Lycopersicum esculentum*) and *L. penelli,* the authors observed that the inheritance of the organelle DNA was probably influenced by the nuclear background of the regenerant. An increase in the percentage of tomato alleles in the nucleus was accompanied by an increased probability of an individual inheriting the tomato chloroplast genome and having more tomato-specific mitochondrial sequences.

Kumar and Cocking (1987) pointed out that the presence of a single parental type mitochondrion in animal somatic hybrid cells (mouse+human) belongs to the parent whose nuclear chromosomes are more stable. They related the propagation of mtDNA from one parent in the somatic hybrids to a particular set of chromosomes.

Earle (1995) mentioned that protoplasts from different tissue types may differ in the number and in the replication rate of the mitochondria they contain. Bonnema et al. (1992) suggested that when suspension cells (growing heterotrophically) are the source of protoplasts, this would

provide a more competitive form of mitochondria. Earle (1995) mentioned a work with *Brassica,* by Landgreen and Glimelius (1990), where there was a preferential transmission of mtDNA from hypocotyl protoplasts. These authors suggested that hypocotyl protoplasts may contain more mitochondria than mesophyll protoplasts because they come from young dividing cells.

Another factor raised by these authors is that leaf mesophyll cells are relatively enriched in plastids when compared to suspension-cultured cells. Kumar and Cocking (1987) mentioned that mesophyll protoplasts have approximately 200 chloroplasts/cell and cell suspension protoplasts possess approximately 20 chloroplasts/cell. The prediction would be that the somatic hybrids would preferentially inherit the mesophyll parent chloroplast DNA. However, the authors did observe a random pattern of inheritance of chloroplast DNA in the symmetric somatic hybrids.

Bonnema et al. (1992) mentioned that non-random organellar inheritance is more frequent in combinations of species that are sexually incompatible. Explanations for non-random inheritance include the tissue source of the protoplasts used in the fusion, intergeneric nucleo-cytoplasmic incompatibility, and differential replication rates for the organelles (Bonnema et al., 1992). Other

factors for plastid inheritance were mentioned by Malone et al. (1992): adequate time for vegetative segregation (even if there is selection for a certain organelle type); the unlimited and disoriented cell divisions in culture; and stringent conditions favoring multiplication of desired organelle population, such as light stimulation and preferential protein biosynthesis.

It is important to remember that techniques of somatic hybridization require that cells and organelles be placed in conditions that are far different from those experienced *in vivo*. Howe (1986), mentioned by Smith (1989), suggested that organelle recombination *in vitro* does not necessarily mean that this phenomenon occurs in nature.

Summary

Further research is necessary to elucidate the mechanisms governing organelle inheritance. Sometimes rules are assumed without questioning the methodologies or all the factors that potentially influence the chloroplast and mtDNA inheritance.

It is interesting to note how chloroplast and mitochondrial genome inheritance can be completely independent events. Chloroplast DNA apparently is inherited in an "organized" and consistent way, with few surprises in

terms of appearance of new configurations in the progeny of a sexual cross or in somatic fusion products. Perhaps this is because recombination does not play a major role in the organization of the chloroplast genome.

Little is known about the plant mitochondrial genome organization. However, considering the array of possibilities (rearrangements, recombination and differential replication) that may occur before, during or after a sexual cross or somatic fusion, there are probably no general rules. Each fusion or cross presents and entirely different situation. In each of these situations, a completely different nuclear genome is formed. This probably plays an important role in determining which mechanisms will be acting during the mitochondrial genome reorganization.

CHAPTER 3
MATERIAL AND METHODS

Plant Material

Sexual Crosses

The intergeneric sexual hybrid family analyzed in this work was developed by Dr. Fred Gmitter at the CREC (Citrus Research and Education Center/University of Florida), Lake Alfred. The seed parent used was LB 1-18, a hybrid of *Citrus reticulata* Blanco cv. 'Clementine' x *C. paradisi* Macf. 'Duncan' grapefruit. Seeds of LB 1-18 are monoembryonic, containing sexually derived zygotic embryos. The sexually compatible pollen parent was a seedling of *Poncirus trifoliata* (L.)Raf., which is no longer extant. Therefore, DNA from *P. trifoliata* cultivar Rubidoux was used for this analysis.

Twenty-six 7 year old progeny from this cross were screened for the inheritance of mitochondria and chloroplast DNA polymorphisms: F1s # 3, 4, 6, 7, 9, 10, 12, 13, 14, 15, 16, 18, 19, 21, 22, 23, 24, 27, 28, 30, 33, 35, 36, 44, 48, 55. F1s # 33 and 44 were open pollinated, and 6 progeny of each one were subjected to the same kind of analysis. Leaf

material from each one of these trees was collected in Lake Alfred (CREC) at different times of the year, but always at about 50% leaf expansion. The leaves were brought to the lab in an iced cooler, and frozen at -80°C.

Somatic Hybrid and Cybrid Combinations

All the somatic hybrid and cybrid combinations listed below were supplied by Dr. Jude Grosser, from CREC (Lake Alfred/FL). These fusions were performed using the PEG method (Grosser and Gmitter, 1990), with the objective of obtaining tetraploid somatic hybrids for breeding purposes. After the fusion, regenerated plants were checked in terms of chromosome number and leaf isozyme banding patterns on starch gels (Chapter 2). As mentioned in chapter 1, although the expected outcome is a symmetric hybrid containing both parental nuclear genomes, some diploid plants resembling the leaf parent were obtained (putative cybrids). Description of the somatic hybrid and cybrid combinations in terms of number of analyzed plants and source of materials is presented in Tables 3.1 and 3.2.

Table 3.1. Description and number (#) of the analyzed somatic hybrid combinations.

EMBRYOGENIC PARENT (suspension cultures)	LEAF PARENT	#
'Succari' sweet orange (*C.sinensis* L.Osbeck)	*Citropsis gilletiana* Swingle & M.Kell.	14
'Succari' sweet orange *(C.sinensis)*	*Atalantia ceylanica* (Arn.)Oliv.	14

Table 3.2. Description and number (#) of the analyzed cybrid combinations.

EMBRYOGENIC PARENT	LEAF PARENT	#
'Willowleaf' mandarin [a] *(C.reticulata* Blanco*)*	'Duncan' grapefruit (*C.paradisi* Macfc.)	4
Swinglea glutinosa [a] Swingle	Sour Orange (*C.aurantium* L.*)*	3
'Rohde Red Valencia' [b] *(C.sinensis* L. Osbeck*)*	'Dancy' mandarin *(C.reticulata* Blanco*)*	5
'Willowleaf' mandarin [a] *(C.reticulata)*	'Valencia' sweet orange *(C.sinensis* L. Osbeck*)*	9
'Hamlin' sweet orange [b] *(C.sinensis* L. Osbeck*)*	'Ponkan' mandarin *(C.reticulata* Blanco*)*	2
Cleopatra mandarin [a] *(C.reticulata* Blanco*)*	Sour Orange *(C.aurantium)*	3

[a] callus

[b] suspension cultures

The leaves were collected from regenerated somatic hybrid or cybrid plants growing in the CREC's greenhouse (Lake Alfred) and brought to Gainesville in an iced cooler. They were kept at -80°C.

The embryogenic suspension cultures and callus were obtained in Lake Alfred, from Dr. Grosser, and were at the same growth stage used at the somatic fusion. The callus was friable, about 2 to 3 weeks old which is "log phase" on a 6 week subculture cycle. The suspensions were about 12 days old in a 14 day subculture cycle.

RFLP Analysis of Nuclear, Chloroplast and Mitochondrial DNA

Total DNA extraction

Total cellular DNA was isolated from 1g of frozen leaf tissue, 1g of embryogenic suspension culture, or 3 g of callus. The same phenol/chloroform extraction method (Durham et al., 1992) was used to obtain DNA from leaves of sexual hybrids and from leaves, callus and suspensions of the parents somatic hybrids and cybrids. The main difference between these different materials was that leaves had to be frozen with liquid nitrogen to be ground. Callus and suspension cells were easily ground without liquid nitrogen.

Restriction Enzyme Digestion and Electrophoresis

The first objective was to find polymorphism between the parents, and then to characterize the F1 hybrids, somatic hybrids, and possible cybrids. With this in mind, total DNA from the sexual and somatic hybrid parents was digested using different restriction enzymes having a six base recognition sequence (*Eco*RI, *Hin*dIII, *Pst*I, *Eco*RV, *Bam*H1, *Sma*I, *Dra*I and *Xba*I) according to the manufacturer's instructions (Life Technologies Inc.). Restriction fragments from 5 ug (for organelle analysis) and 20 ug (for nuclear analysis) of total DNA were separated by electrophoresis through a 0.8% agarose gel in TPE buffer (300mM NaH_2PO_4, 360mM Trizma base and 10mM $Na_2EDTA.2H_2O$) at 40v for 20 hours. The size of the gel was 380 cm^2.

Southern Blotting and Hybridization

These DNA fragments were blotted to Hybond-N supports (Amersham Corporation) by capillary transfer (Sambrook et al., 1989) and hybridized with radiolabeled mitochondrial, chloroplast and nuclear probes. The blots were pre-hybridized for at least 1 hour in 10xSSPE (1x SSPE = 1.8M NaCl, 0.1M NaH_2PO_4, 0.01M Na_2EDTA), 50x Denhardts (50x = 1% w/v Bovine Serum Albumin, 1% w/v Ficoll, 1% w/v PVP 360) and 10% w/v SDS. To avoid background in the autoradiographs, herring sperm DNA (500 ug of DNA) was denatured by boiling for 10

min., chilled on ice and added to the prehybridization solution.

The probes described in table 3.4 were radiolabeled with alpha ^{32}PdCTP through random priming (Feinberg and Vogelstein, 1984), using a "Gibco BRL Random Primers DNA Labeling System." Following denaturation, the probes were added directly to the prehybridization solution, and the blots were hybridized for a minimum of 16 hours, at 60°C.

The blots were washed after hybridization in a 2x SSPE, 0.1% SDS solution at room temperature for 10 minutes. Following a second 2x SSPE, 0.1% SDS wash for 10 min. at room temperature, the blots were washed in 1x SSPE, 0.1% SDS at 60°C for 15 min. A final wash in 0.1x SSPE, 0.1%SDS was done at 60°C for 10 min. Membranes were exposed to Kodak (X-Omat RP XRP-5) film for 7-14 days.

Hybridization Probes

Tables 3.3 and 3.4 describe the heterologous probes used for both the sexual cross and somatic hybrid analyses. The *26SrRNA* cDNA was constructed by Mr. Byoung Kim, at Dr. Chase's lab, using reverse transcriptase PCR (RT-PCR) techniques. The original *cytf* clone was supplied by Dr. Ken Kline (Horticultural Sciences Dept./Gainesville). Plasmid culture from a citrus lycopene cyclase clone was provided by Mr. John Melton, in Dr. Gloria Moore's lab.

Table 3.3 Source of the genomic and mitochondrial heterologous probes.

ORIGIN	GENE	DESCRIPTION	SIZE	ENZYME	INSERT RECOVERY METHOD	SOURCE
citrus nuclear	*lyc. cyc.*	Lycopene cyclase (cDNA)	1.1 kb	*SphI/ PstI*	PCR from plasmid DNA	G.A.Moore
soybean nuclear	*18S rRNA*	18S ribosomal RNA (internal coding)	1.1 kb	*EcoRI/ BamHI*	plasmid insert	Eckenrode et al., 1985
Zea mays mitochondria	*cob*	cytochrome b (internal coding)	1.2 kb		PCR from mt DNA	this study
Phaseolus vulgaris mitochondria	*atpA*	alpha-subunit of the F1 ATPase (internal coding - Genomic Clone)	0.9 kb	*EcoRI/ Bam*H1	PCR from plasmid DNA	Chase and Ortega, 1992
Z. mays mitochondria	*coxI*	Cytochrome Oxidase I (internal coding)	1.4 kb		PCR from mt DNA	this study
Z. mays mitochondria	*coxII*	Cytochrome Oxidase II (internal coding)	1.4 kb		PCR from mt DNA	this study
Z. mays mitochondria	*coxIII*	Cytochrome Oxidase III (internal coding)	0.5 kb		PCR from mt DNA	this study
Sorghum bicolor mitochondria	*atp9*	subunit of ATP synthase (cDNA)	0.6 kb	*Pvu*II	plasmid insert	Salazar et al., 1991
P. vulgaris mitochondria	*26S rRNA*	26S ribosomal RNA (internal coding-cDNA)	0.6 kb	*PvuII*	PCR from plasmid DNA	this study

Table 3.4 Source of the chloroplast heterologous probes.

ORIGIN	GENE	DESCRIPTION	SIZE	ENZYME	RECOVERY	SOURCE
spinach chloroplast	*petD*	subunit 4 of cytochrome b_6f complex (genomic clone)	0.4 kb	*BamHI*	plasmid insert	Zurowski et al,1981
spinach chloroplast	*rbcl*	RUBISCO large subunit (genomic clone)	1.2 kb	*PstI/ EcoRI*	plasmid insert	Zurowski et al,1981
pea chloroplast	*cytf*	cytochrome f (internal coding - cDNA)	1.2 kb	*SstI/ XbaI*	plasmid insert	Willey et al., 1984
mung bean chloroplast	cp1	23S ribosomal RNA 3'region (genomic clone)	9.7 kb	*PstI*	plasmid insert	Palmer et al., 1981a
mung bean chloroplast	cp2	16S ribosomal RNA 3'region (genomic clone)	11 kb	*PstI*	plasmid insert	Palmer et al., 1981a
tobacco chloroplast	cp3	petD rpoA region (genomic clone)	4.7 kb	*SstI/ SalI*	PCR from chloroplast DNA	Palmer et al., 1981b

PCR Reaction

The same PCR protocol was used in the production of the *lyc.cyc., atpA, cob, coxI, coxII, coxIII* and cp3 probes and is described in Tables 3.5 and 3.6.

Table 3.5. PCR amplification reaction.

Target DNA	2ul
Primer 1(10ng/ul)	10ul
Primer 2(10ng/ul)	10ul
HPLC water	65ul
10 x Taq buffer[x]	10ul
2.5 mM Mg beads	1
dNTPs[y] (2.5mM)	2ul
Taq polymerase[x]	1ul
Total Volume	100ul

[x] supplied by Promega Corp.

[y] dNTPs = deoxyribonucleoside triphosphates

Table 3.6. PCR Program.

STEP	TEMPERATURE/TIME
1	94°C/1 min.
2	55°C/2 min
3	72°C/3 min
4	Go to step 1-3, 29 more times
5	10°C

Primer Sequences

The primers used in PCR reactions are described in Table 3.7.

Table 3.7. Primer sequences.

PROBES	PRIMER	SEQUENCE
lyc.cyc., *atpA*, and cp3	NEB1211[X]	5'dGTA.AAA.CGA.CGG.CCA.GT 3'
	CC33[Y]	5'CAG.GAA.ACA.GCT.ATG.ACC 3'
cob	CC52[Y] (sense)	5'CTA.TTC.CGT.GTA.ATA.TTT.TGG 3'
	CC53[Y] (antisense)	5'ATG.ACT.ATA.AGG.AAC.CAA.CG 3'
coxI	CC79 [Y] (sense)	5'GGT.CCG.ATG.GCT.CTT.CTC 3'
	CC80 [Y] (antisense)	5'GAT.AGT.TGG.AAG.TTC.TCC 3'
coxII	CC81 [Y] (sense)	5'GCG.GAA.CCA.TGG.CAA.TTA 3'
	CC82 [Y] (antisense)	5'GGC.ATG.ATT.AGT.TCC.ACT 3'
coxIII	CC66 [Y] (sense)	5'GTA.GAT.CCA.AGT.CCA.TGG 3'
	CC67 [Y] (antisense)	5'GCA.TGA.TGG.GCC.CAA.GTT 3'

[X] supplied by New England Biolabs

[Y] supplied by DNA Synthesis Core ICBR/University of Florida

NEB1211 and CC33 hybridize to plasmid DNA sequences. In contrast, CC52, CC53, CC79, CC80, CC81, CC82, CC66 and CC67 primers were designed based on the mitochondrial *cob* (Dawson et al., 1984), *coxI* (Isaac et al., 1985), *cox II* (Moon et al., 1985) and *coxIII* (Malek et al., 1996) published

sequences.

Transformation Techniques

The following transformation protocol was used in Dr. Chase's lab to maintain the *cytf* and *26SrRNA* cloned DNAs obtained from other investigators:

1. Thaw 100 ml of DH5alpha competent cells (Life Technologies, Inc.) and mix gently.

2. Dispense to pre-chilled 4 ml snap-cap tubes.

3. Add 2ul of DNA and swirl gently to mix.

4. Incubate on ice for 30 min.

5. Heat pulse at 42°C for 55 sec.

6. Add 400 ul room temperature LB (1% bactotryptone, 0.5% yeast extract, 0.5% sodium chloride and 1.2% Difco agar), swirl gently to mix.

7. Spread over LB plates (with 0.1M IPTG, 20mg/ml ampicillin and 2%w/v X-gal) for control over transformers.

8. Allow media to soak into plates. Incubate upside down at 37 °C overnight.

9. In theory, only the white colonies are transformed, but our experience showed that sometimes blue colonies might harbor the insert.

10. Select single white colonies (and occasionally

blue) and proceed to a plasmid prep, for DNA recovery.

Plasmid Prep

1. Inoculate each clone into 3ml of LB plus ampicillin (20 mg/ml), at 37 °C, with continuous shaking, for 12-18 hours.

2. Prepare fresh triton lysis (1M Tris pH8, 0.2M EDTA pH8, 0.1% Triton X100 and 94% distilled water) and 10 mg of lysozyme/ml of ST (50mM Tris pH8, 25% sucrose in 100 ml of distilled water). Keep lysozyme on ice and triton lysis at room temperature.

3. Transfer 1.5ml of each culture to Eppendorf tubes.

4. Spin 45 sec. at 12,000 rpm in microfuge (Eppendorf 6415C).

5. Discard supernatant and drain tubes upside down.

6. Place tubes on ice and add 38 ul of ice-cold ST to each tube.

7. Vortex until the cells are completely resuspended.

8. Add 13 ul of fresh lysozyme solution to each tube.

9. Mix gently and incubate on ice for 5 min.

10. Add 32 ul of 0.2M EDTA, pH 8.

11. Mix gently and incubate on ice for 5 min.

12. Add 80ul of triton lysis buffer to each tube; tilt tube gently one time to mix.

13. Incubate on ice for 15 min.

14. Spin in microfuge top speed for 20 min.

15. Discard pellets and pour off supernatant into clean microfuge tube.

16. Add 160 ul of water-saturated phenol, close tubes and vortex off and on for 5 min.

17. Remove top phase to clean tube.

18. Add 16ul of 8M ammonium acetate and 320ul of absolute ethanol. Mix.

19. Incubate at -20°C, overnight.

20. Spin 10 min in microfuge to pellet DNA.

21. Pour off supernatants and fill tubes with 70% ethanol; incubate on ice for 15 min.

22. Spin 5 min. in microfuge to repellet (cold room).

23. Pour off supernatant carefully.

24. Briefly air dry pellets, before resuspending in 60ul of sterile 0.1X NTE (0.01M Tris, 0.001M Na_2EDTA, 0.01M NaCl, at pH 8) by pipetting.

25. Assay 20 ul of each prep by agarose gel electrophoresis.

26. Proceed to Insert Recovery.

Insert Recovery

All probes were recovered using the following "Insert Recovery" protocol:

1. Run digested clones or PCR products on a 0.8% agarose gel in TAE buffer (1X TAE = 0.04M Tris, 0.001M EDTA, 0.11 % glacial acetic acid, pH 7.8).

2. Stain gel for 10 minutes in ethidium bromide.

3. Transfer gel to light box, quickly excise bands and transfer to Eppendorf tubes (already perforated 5 times, in the bottom, with a 16 gauge needle).

4. Introduce each Eppendorf in a 5cc syringe, with large pieces of silanized glass wool packed in the bottom.

5. Transfer the whole apparatus (Eppendorf inside syringe)to a 11cc dispo receiving tube.

6. Spin the dispo tubes in a SA600 rotor (Centrifuge Superspeed Sorvall RC2-B), 4,000 rpm, 10min.

7. Transfer liquid from bottom of receiving tube to an eppendorf tube.

8. Estimate volume. Add 1/10 volume of 8M ammonium acetate and 2 volumes of absolute ethanol. Mix well.

9. Incubate -20°C overnight.

10. Pellet DNA in microfuge at top speed for 20 minutes in cold room.

11. Pour off supernatant, fill tube with 70% ethanol and incubate on ice for 10 minutes.

12. Repellet in microfuge at top speed for 5 min. in cold room.

13. Pull off the supernatant with a pipette. Repellet in microfuge for 30 seconds and pull remaining supernatant with a fine pipet tip.

14. Resuspend pellet in 20 ul of sterile distilled water.

15. Read the OD of a 1/50 dilution at 260nm and 280nm.

RAPDs

The progeny from F1s 33 and 44 from the sexual cross were analyzed with random amplified polymorphic DNA markers (RAPDs), in order to obtain confirmation of their zygotic origin. These analysis were performed by Mr. Huang Shu in Dr. Gmitter's lab at the CREC, Lake Alfred.

PCR Reaction

The components for the reactions and the PCR amplification program used for the RAPDs analysis were

described by Gmitter et al. (1996). The primer that best characterized the outcross progeny of F1s 33 and 44 was "H01" supplied by Operon Technologies; its sequence is 5'GGTCGGAGAA 3'.

DNA Copy Number Analysis

Total DNA from callus, suspension, or leaves was used to estimate the organelle input from each kind of plant material used as a parental source in somatic hybridization experiments. RFLP analysis of these materials proceeded for a cybrid (Sour Orange + Cleopatra) and a somatic hybrid ('Succari'+ *Atalantia ceylanica*) combination. DNA samples of 15 ug were digested with restriction enzymes, fractioned by agarose gel electrophoresis, and transferred to nylon supports. The same blot was hybridized with radiolabeled *lyc.cyc., cob* and *cytf* probes. The citrus nuclear *lyc.cyc.* was used as control for the amount of total DNA loaded for each of the plant materials. The *cob* and *cyt f* clones were used to examine the mitochondria and chloroplast input, respectively. Densitometry analysis of each RFLP band, based on the "Integrated Density Value", was performed with an IS-1000 Digital Imaging System (Alpha Innotech Corporation).

CHAPTER 4
RESULTS

Sexual Cross

DNA Polymorphisms Among the Parents

To assess DNA polymorphisms between parents, total cellular DNA from each parent was digested and hybridized with two chloroplast probes, *cytf* and *petD* and six mitochondrial probes *atpA*, *coxI*, *coxII*, *coxIII*, *26SrRNA* and *atp9*. Due to the fact that the male parent is no longer extant, two different varieties of *P. trifoliata* (Gainesville and Rubidoux) were examined in order to compare their banding patterns (Fig 4.1). No difference was observed between these two varieties with all the probe/restriction enzyme combinations, and Rubidoux was chosen as a DNA substitute for the male parent.

Most hybridization profiles revealed polymorphisms between the paternal (*P. trifoliata*) and maternal (LB 1-18) mtDNA (Table 4.1). In some cases, the *P. trifoliata* configurations were composed of abundant unique bands plus low abundance configurations matching those of LB 1-18.

Table 4.1. Probe/enzyme Combinations Demonstrating Polymorphisms Between Parents.

PROBE	ENZYMES
atpA	*EcoRV/HindIII/BamHI/EcoRI*
coxI	*EcoRV/BamHI/PstI/XbaI*
coxII	*HindIII/EcoRI*
coxIII	*HindIII/SstI*
26SrRNA	*SmaI*
cytf	*BamHI/EcoRI/XbaI*
petD	*HindIII*

Hybridizations of the *atp9* mitochondrial clone to *EcoRV, HindIII, BamHI, EcoRI* and *PstI* digested DNAs did not reveal any useful polymorphism between the parents. An interesting pattern was observed when *EcoRV* digested DNAs were hybridized with this mitochondrial clone. There was a distinct 11 kb fragment characteristic of the male parent and a 14 kb fragment characteristic of the female parent. However, the 14 kb maternal fragment can be also visualized at low abundance in the male parent (Fig. 4.1).

Seven polymorhic probe-enzyme combinations were selected for use in this inheritance analysis. Table 4.2 presents the size of the paternal and maternal fragments, revealed by hybridization with each of the heterologous probes.

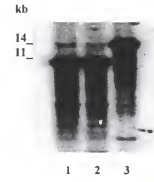


Fig 4.1. Autoradiograph of Southern blot showing mtDNA of LB 1-18 (maternal parent - lane 3) and *P. trifoliata* (paternal parent - Rubidoux lane 1 and 'Gainesville' lane 2). Total DNA was digested with *EcoRV* and hybridized to an *atp9* coding region clone.

Table 4.2. Fragments Characteristic of the Female and Male Parents.

PROBE	ENZYME	*P.trifoliata*	LB 1-18
atpA	*HindIII*	4.3 kb	3.4 kb
coxI	*PstI*	6.5 kb	10 kb
coxII	*EcoRI*	16 and 2.6 kb	9 and 2.6 kb
coxIII	*HindIII*	6 kb	4.5 kb
26SrRNA	*SmaI*	10 and 4.5 kb	18 and 10 kb
cytf	*BamHI*	10 kb	13 kb
petD	*HindIII*	5 kb	11 and 9.5 kb

Mitochondrial DNA Inheritance

Some mitochondrial loci appeared to be biparentally inherited in the sexual cross. Hybridizations of the *atpA* mtDNA probe to *Hin*dIII digests of the 26 F1 plants showed the intense 3.4 kb maternal fragment in all the progeny plants. However, 17 F1 plants (F1s # 6, 7, 9, 10, 12, 13, 14, 15, 16, 18, 23, 27, 28, 30, 33, 36 and 44) showed a

faint 4.3 kb fragment characteristic of the paternal *P.trifoliata* parent. Fig 4.2-A shows 14 of the 26 progeny, where 8 F1s (lanes 4,5,6,7,8,11,12,14) exhibited the paternal configuration. The *coxII* probe identified the 9.0 and 2.6 kb maternal fragments in *EcoRI* digests from all F1 plants. Again, the same 17 F1 hybrids carried a faint 16 kb paternal fragment (Fig 4.2B). A similar result was observed when total DNA of the 26 F1 hybrids was hybridized to the *coxIII* clone. All hybrids presented the strong maternal 4.5 kb fragment and the same 17 F1 hybrids presented a faint 6 kb fragment, characteristic of *P. trifoliata* (Fig 4.2C). In summary, the same F1 hybrids carried the paternal configurations for all three loci. These three loci appeared to be "linked" in the same seventeen progeny that carried the *P. trifoliata* configurations, whereas the other 9 progeny carried none of the paternal configurations. The intergeneric hybrids are therefore segregating for the presence or absence of the paternal mtDNA configurations.

However, this pattern of inheritance was not observed for all mitochondrial loci. Additional results were obtained using the same 26 hybrids and the *coxI* and *26rRNA* clones. *CoxI* identified only the maternal 10 kb fragment in these sexual hybrids, with no presence of the paternal 6.5 kb in any of the 26 sexual hybrids. In the Fig. 4.3A, the same 14 F1 hybrids showed in the previous figures presented

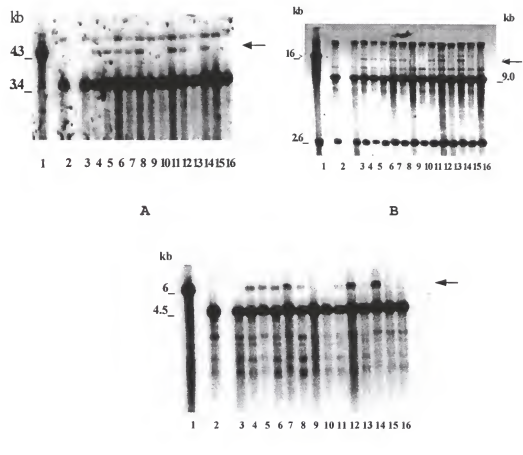


Fig 4.2. Paternal mtDNA configurations in the progeny of an intergeneric *Citrus* x *Poncirus* Cross. Autoradiographs of Southern blots showing mtDNA of 14 of the F1 hybrids of LB 1-18 (maternal parent - lane 2) x *P. trifoliata* (paternal parent - lane 1). F1 hybrids are shown in lanes 3-16 (hybrids # 3, 6, 7, 10, 15, 16, 19, 21, 27, 33, 35, 44, 48 and 55). Enzyme-probe combinations are as follows: panel A, *HindIII-atpA;* panel B, *EcoRI*-coxII; panel C, *HindIII-coxIII*. The arrows mark bands possibly transmitted to the progeny through the paternal parent.

a straight maternal inheritance of the *coxI* locus. A similar result was observed when total DNA of the 26 F1 hybrids was digested with *SmaI* and hybridized to the *26SrRNA* clone. Only the maternal bands (10 and 18 kb) were present in the F1 hybrids (Fig 4.3B). Therefore, the observation of paternal mtDNA configurations was not necessarily true for all loci tested. However, because we are substituting *P. trifoliata* Rubidoux for the original paternal parent, we cannot exclude the possibility that this parent lacked the polymorphic bands observed in Rubidoux.

In order to assess the consequences of paternal mtDNA configurations for subsequent plant generations, the outcross progeny of F1 #'s 33 and 44 (which carried the paternal configurations) were analyzed using the mitochondrial *atpA* coding region clone. Two of six outcross progeny from F1 # 33 carried the 4.3 kb *P. trifoliata* configuration (Fig 4.4A). Three of six outcross F1 # 44 progeny have the 4.3 kb band (Fig 4.4B). Therefore, paternal mtDNA configurations persisted into the next generation.

RAPDs

RAPDs were the chosen method of analysis to quickly prove the zygotic origin of the outcross progeny from F1#33 and #44. These analysis were performed by Mr. Huang Shu,

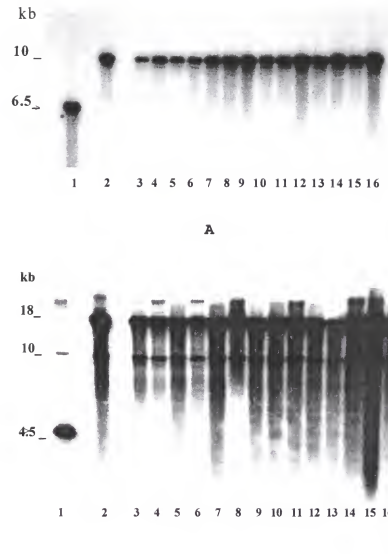
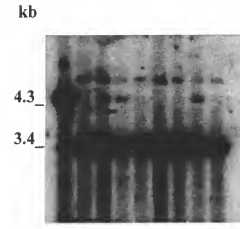


Fig 4.3. Autoradiograph of Southern blot showing mtDNA of the intergeneric hybrids of LB 1-18 (maternal parent - lane 2) x *P. trifoliata* (paternal parent - lane 1). Fourteen F1 hybrids are shown in lanes 3-16 (hybrids # 3, 6, 7, 10, 15, 16, 19, 21, 27, 33, 35, 44, 48 and 55). Enzyme-probe combinations are as follows: panel A, *PstI-coxI;* panel B, *SmaI-26SrRNA.*



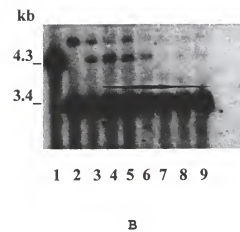
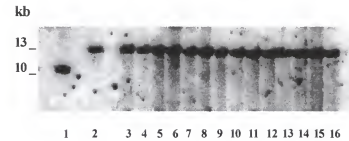


Fig 4.4. Autoradiograph of Southern blot showing mtDNA of outcross progeny from intergeneric hybrid #33 (panel A, lane 3) and #44 (panel B, lane 3). Six progeny are shown in lanes 4-9. LB 1-18 (grand maternal parent - lane 2), *P. trifoliata* (grand paternal parent - lane 1). Total DNA was digested with *HindIII* and hybridized to the *atpA* clone.

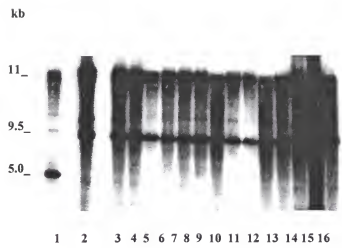
working in Dr. Fred Gmitter's lab at the UF/IFAS CREC at Lake Alfred,FL. Among all the primers tested, H01 was the one that gave the more conclusive results indicating the outcross progenies of F1 # 33 and # 44 as segregating zygotic populations.

Chloroplast DNA Inheritance

In contrast to mtDNA inheritance, chloroplast DNA appeared to exhibit strict maternal inheritance. The *cytf* probe detected the maternal 13 kb *Bam*H1 fragment in all the 26 F1 progeny. No paternal (10 kb) fragments were observed in any of the hybrids (Fig 4.5). Only LB 1-18 configurations (9.5 and 11 kb fragments) were observed in all the F1 hybrids analyzed, after total DNA was digested and hybridized to the chloroplast *petD* clone. Although the lack of polymorphisms limited the number of loci that could be tested, the results indicated a maternal inheritance of chloroplasts. Again, we cannot exclude the possibility that the original paternal parent lacked the polymorphic bands observed in *P. trifoliata* Rubidoux.



A



B

Fig 4.5. Maternal inheritance of chloroplast genome in the intergeneric hybrids. Autoradiograph of Southern blot showing chloroplast DNA of the intergeneric hybrids of LB 1-18 (maternal parent - lane 2) x *P. trifoliata* (paternal parent - lane 1). Fourteen F1 hybrids (hybrids # 3, 6, 7, 10, 15, 16, 19, 21, 27, 33, 35, 44, 48 and 55) are shown in lanes 3-16 . Enzyme-probe combinations are as follows: panel A, *BamH1-cytf;* panel B, *HindIII-petD.*

Summary

There was an apparent transmission of the mitochondrial *atpA, coxII* and *coxIII* paternal configurations to 17 of the 26 sexual hybrids of LB 1-18 x *P. trifoliata*. These paternal configurations also segregated in the outcross progeny of two F1 hybrids. However, there was an apparent straight maternal inheritance of the mitochondrial *coxI* and *26SrRNA* in the 26 sexual hybrids. Apparent maternal inheritance of chloroplast DNA was observed in this sexual cross for the *cytf* and *petD* loci.

Somatic Fusions

DNA Polymorphisms Distinguishing the Parents Organelle Genomes

The first objective was to find polymorphisms that would distinguish the two somatic fusion parents. This was problematic, especially concerning the chloroplast genome. Chloroplast gene coding probes (such as *cytf* and *petD)* did not show any polymorphisms among the somatic fusion parents. Longer chloroplast probes that included both coding and noncoding sequences were the solution, revealing polymorphisms among these parents. The mitochondrial *atpA* probe was used to evaluate the mtDNA inheritance in these hybrids. For chloroplast inheritance cp1, cp2 and cp3 were

the clones that revealed the necessary polymorphisms. In order to discriminate between somatic hybrids and cybrids, two genomic probes were used: *18SrRNA* and *lycopene cyclase* (*lyc.cyc*). No genomic polymorphism was observed during this research for the 'Succari' + *Atalantia ceylanica* and the 'Hamlin' + 'Ponkan' combinations. However, Grosser et al. (1996b) had previously used RAPD markers to characterize the nuclear genome of the 'Succari' + *A. ceylanica* and the 'Succari' + *Citropsis gilletiana* fusion products. Tables 4.3, 4.4 and 4.5 present the sizes of the parental (leaf and embryogenic) DNA restriction fragments from the nuclear, mitochondrial, and chloroplast genomes, respectively.

Table 4.3. DNA Restriction Fragments Revealed after Hybridization with Nuclear-Encoded Probes.

PROBE	ENZYME	EMBRYOGENIC PARENT	LEAF PARENT
lyc. cyc.	*EcoRV*	'Willowleaf' mandarin (4.5 kb)	'Duncan' grapefruit (3 and 4 kb)
lyc. cyc.	*HindIII*	*Swinglea glutinosa* (3.5 kb)	Sour Orange (1.8 kb)
lyc. cyc.	*EcoRV*	'Rohde Red Valencia' (4.5 and 5 kb)	'Dancy' mandarin (5 kb)
18SrRNA	*EcoRI*	'Willowleaf' mandarin (6.5 and 8 kb)	'Valencia' sweet orange (6.5 and 7.5 kb)
18SrRNA	*EcoRI*	Cleopatra mandarin (6.5 and 8 kb)	Sour Orange (6.5 and 9.5 kb)

Table 4.4. DNA Restriction Fragments Revealed by Hybridization with the Mitochondrial *atpA* Clone.

ENZYME	EMBRYOGENIC PARENT	LEAF PARENT
PstI	'Succari' sweet orange (2.5, 3.5, 6 and 7.5kb)	*Citropsis gilletiana* (2 and 2.8 kb)
PstI	'Succari' sweet orange (2.5, 3.5, 6 and 7.5kb)	*Atalantia ceylanica (14 and 5 kb)*
EcoRV	'Willowleaf' mandarin (16 kb)	'Duncan' grapefruit (5, 8, 22 and 25 kb)
HindIII	*Swinglea glutinosa* (2.5, 6.5, 8 and 11 kb)	Sour Orange (1, 6.5 and 8 kb)
EcoRV	'Rohde Red Valencia' (4.5, 7, 19 and 20 kb)	'Dancy' mandarin (16 kb)
HindIII	'Willowleaf' mandarin (3.2 kb)	'Valencia' sweet orange (2.5, 5.5, 6 and 9 kb)
HindIII	'Hamlin' sweet orange (2.5, 5.5, 6 and 9 kb)	'Ponkan' mandarin (3.2 kb)
EcoRV	Cleopatra mandarin (4, 6, 6.5 and 8 kb)	Sour Orange (4.5 and 7.5 kb)

Table 4.5. DNA Restriction Fragments Revealed by Hybridization with Chloroplast Encoded Clones.

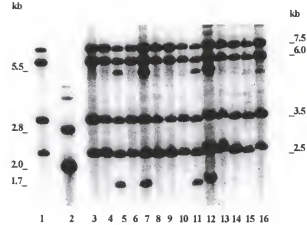
PROBE	ENZYME	EMBRYOGENIC PARENT	LEAF PARENT
cp2	*HindIII*	'Succari' sweet orange (5, 6, 8.5, 11 and 14 kb)	*Citropsis gilletiana* (5, 6, 7.5, 11 and 14 kb)
cp3	*PstI*	'Succari' sweet orange (7 kb)	*Atalantia ceylanica* (4.5 kb)
cp1	*BamHI*	'Willowleaf' mandarin (4 and 5 kb)	'Duncan' grapefruit (3.5 and 5 kb)
cp1	*BamHI*	*Swinglea glutinosa* (4.5 and 6 kb)	Sour Orange (4 and 6 kb)
cp3	*PstI*	'Rohde Red Valencia' (6.5 kb)	'Dancy' mandarin (1.5 and 4.5 kb)
cp1	*BamHI*	'Willowleaf' mandarin (4 and 5 kb)	'Valencia' sweet orange (3.5 and 5 kb)
cp3	*PstI*	'Hamlin' sweet orange (7 kb)	'Ponkan' mandarin (4.5 kb)
cp1	*BamHI*	Cleopatra mandarin (4 and 5 kb)	Sour Orange (3.5 and 5 kb)

Nuclear and Organelle Inheritance

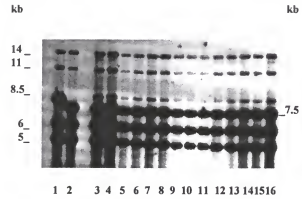
Plants from the eight somatic fusion experiments were analyzed to determine their nuclear, mitochondrial and chloroplast genome composition. Results are provided in the following pages. When analyzing the results with respect to the nuclear genome, it is important to take into account that somatic hybrids are tetraploid and that cybrids are diploid plants. Therefore, considering the definition stated in Chapter 2, somatic hybrids should have all the parental configurations and cybrids should match one of the parental configurations. In terms of organelle inheritance, as reviewed in Chapter 2, there is a consensus in the literature that citrus somatic hybrids and cybrids inherit their mitochondrial genome from the embryogenic parent. In terms of the chloroplast genome, the results demonstrate a random pattern of inheritance.

'Succari'+ *Citropsis gilletiana*

All the hybrids produced in this combination were verified to be somatic hybrids by Grosser et al. (1996b), using RAPD markers. In terms of the mitochondrial genome (Fig 4.6), all the 14 somatic hybrids inherited their mtDNA from 'Succari' (embryogenic parent), although some "novel" (non parental) bands can be observed in DNA from some of the



A



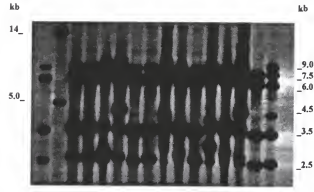
B

Fig 4.6. Autoradiograph of Southern blot showing mtDNA (A) and chloroplast DNA (B) of the somatic hybrids of 'Succari' (embryogenic parent - lane 1) + *Citropsis gilletiana* (leaf parent - lane 2). Fourteen somatic hybrids are shown in lanes 3-16. Enzyme-probe combinations are as follows: panel A, *PstI-atpA;* panel B, *HindIII*-cp2.

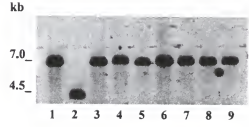
regenerants (Fig 4.6A). A very interesting result was obtained in terms of the chloroplast genome inheritance in this combination: all the somatic hybrids presented both parental chloroplast genomes, which is a very unusual result (Fig 4.6B). The origin of the non-parental mitochondrial bands and the chloroplast inheritance in this combination will be discussed further in Chapter 4.

'Succari' + *Atalantia ceylanica*

Analysis of the 'Succari' + *A. ceylanica* combination with the *lyc.cyc.* and *26SrRNA* clones and more than 6 different restriction enzymes revealed no genomic polymorphism between the parents. However, the 14 hybrids analyzed in this research (Fig 4.7) were verified to be somatic hybrids by Grosser et al. (1996b) using RAPDs. Again the mtDNA was inherited from the embryogenic parent, with the presence of "novel" bands in some of the somatic hybrids. However, these non-parental bands may be due, in part, to incomplete DNA digestion. The chloroplast genome was also inherited from the embryogenic parent (although, in this case, only seven of the 14 somatic hybrids were analyzed).



A



B

Fig 4.7. Autoradiograph of Southern blot showing mtDNA (A) and chloroplast DNA (B) of the somatic hybrids of 'Succari' (embryogenic parent - lane 1) + *Atalantia ceylanica* (leaf parent - lane 2). Somatic hybrids are shown in lanes 3-16 (panel A) and 3-9 (Panel B). Enzyme-probe combinations are as follows: panel A, *PstI-atpA;* panel B, *PstI*-cp3.

'Willowleaf' + 'Duncan'

The four products of the 'Willowleaf' + 'Duncan' fusion were confirmed cybrids when the *lyc.cyc.* genomic probe was used. The cybrids RFLP pattern matched the leaf parent's ('Duncan') configuration, not presenting the 4.5 kb band characteristic of the 'Willowleaf' (embryogenic parent) genome. The mitochondrial genome was inherited from 'Willowleaf', again with the presence of a "novel band" (9.5 kb). The chloroplast genome in this cybrid combination was inherited in a random fashion, with 1 cybrid presenting the 'Willowleaf' chloroplast pattern and 3 cybrids inheriting the 'Duncan' chloroplast genome (Fig. 4.8).

Swinglea glutinosa + Sour Orange

Three putative cybrids of *S. glutinosa* + Sour Orange were analyzed in terms of their nuclear, mitochondrial and chloroplast genomes (Fig 4.9). They presented the Sour Orange (leaf parent) genomic configuration, without the 3.5 kb band characteristic of *S. glutinosa* (embryogenic parent), as expected in cybrid plants. The mitochondrial genome was apparently inherited from the embryogenic parent. However, the presence in the cybrids of multiple non-parental bands resulted in difficulty of reaching an accurate interpretation. Apparently the cybrids inherited the 8, 6.5

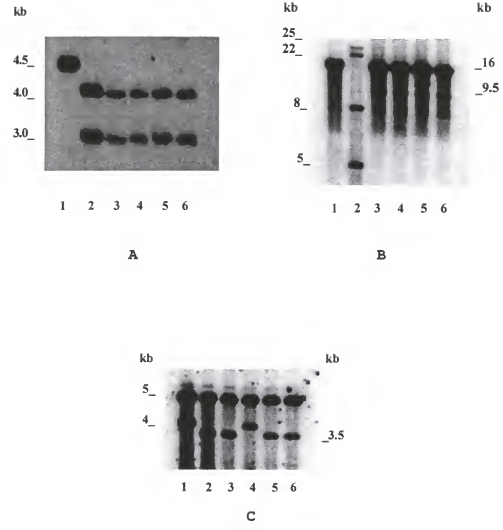


Fig 4.8. Autoradiograph of Southern blot showing genomic DNA (A), mtDNA (B) and chloroplast DNA (C) of the cybrids of 'Willowleaf' (embryogenic parent - lane 1) + 'Duncan' (leaf parent - lane 2). Four cybrids are shown in lanes 3-6. Enzyme-probe combinations are as follows: panel A, *EcoRV-lyc.cyc.;* panel B, *EcoRV-atpA;* panel C, *BamHI-* cp1.

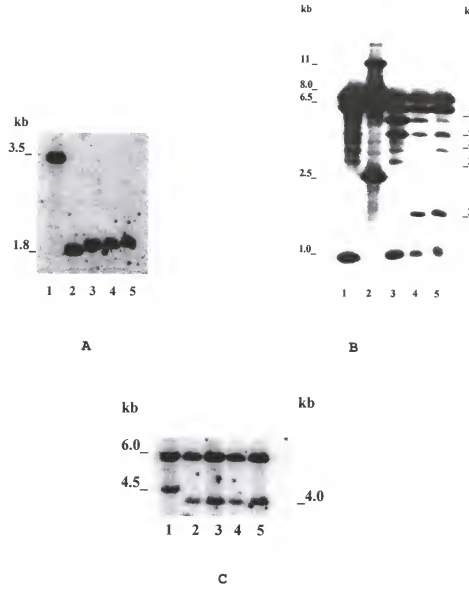
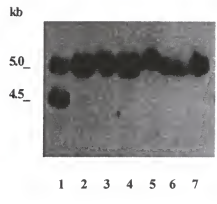


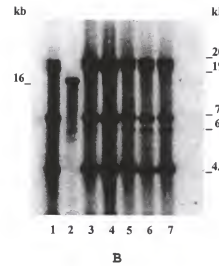
Fig 4.9. Autoradiograph of Southern blot showing genomic DNA (A), mtDNA (B) and chloroplast DNA (C) of the cybrids of *Swinglea glutinosa* (embryogenic parent - lane 1) + Sour Orange (leaf parent - lane 2). Three cybrids are shown in lanes 3-5. Enzyme-probe combinations are as follows: panel A, *HindIII-lyc.cyc.;* panel B, *HindIII-atpA;* panel C, *BamHI-*cp1.

and 1.0 kb bands of the embryogenic parent and did not present the 11 and 2.5 kb bands observed in the leaf parent. Non parental bands (1, 2, 3, 3.5, 4.5 and 5.5 kb) could also be hypothesized to be due to partial total DNA digestion. All the cybrids inherited their chloroplast genome from the leaf parent.

'Rohde Red Valencia' + 'Dancy'

Five seedlings produced by the fusion of 'Rohde Red Valencia' and 'Dancy' were proved cybrids by the *lyc.cyc.* probe. The seedlings' genomic configurations matched that of the leaf parent ('Dancy'), not presenting the 4.5 kb band characteristic of 'Rohde Red Valencia'. Apparently the cybrids inherited their mitochondrial genome from the embryogenic parent ('Rohde Red Valencia'), although all presented a 6.5 kb band that could be visualized (Fig 4.10) in very low amounts, in the 'Dancy' genome. Perhaps what could at first be considered as a "novel" band (6.5 kb) is, in reality, a small contribution of the leaf parent's mitochondrial genome. Another possibility is that this 6.5 kb band is also present in the 'Rohde Red Valencia' mitochondrial genome, and cannot be easily visualized in the autoradiograph (Fig 4.10). The cybrids presented a completely random inheritance of the chloroplast genome: two





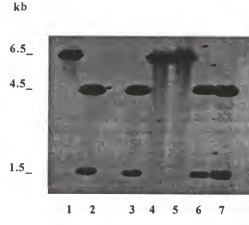


Fig 4.10. Autoradiograph of Southern blot showing genomic DNA (A), mtDNA (B) and chloroplast DNA (C) of the cybrids of 'Rohde Red Valencia' (embryogenic parent - lane 1) + 'Dancy' (leaf parent - lane 2). Five cybrids are shown in lanes 3-7. Enzyme-probe combinations are as follows: panel A, *EcoRV-lyc.cyc.;* panel B, *EcoRV-atpA;* panel C, *PstI-* cp3.

the embryogenic parent and three presented the leaf parent's chloroplast genome.

'Willowleaf'+ 'Valencia'

The cytoplasmic *18SrRNA* clone confirmed the cybrid nature of the nine analyzed seedlings. The cybrids did not present the 8kb genomic band characteristic of the 'Willowleaf' (the embryogenic parent). The mitochondrial genome was inherited from the embryogenic parent with no "novel bands" observed. The chloroplast genome was again randomly inherited: eight cybrids presented the embryogenic parent chloroplast configuration and just one inherited the leaf parent ('Valencia') chloroplast genome (Fig. 4.11).

'Hamlin'+ 'Ponkan'

Despite attempts to find genomic polymorphism between these two parents, no success was achieved with the *lyc.cyc.* and *18SrRNA* probes. However, the 2 regenerants analyzed present morphological features of 'Ponkan' (leaf parent) and diploid chromosome number, characteristic of cybrid plants (J.W.Grosser, personal communication). Regarding the mitochondrial genome, the two possible cybrids inherited the embryogenic parent's ('Hamlin') mtDNA with no "novel" bands observed. In this particular case, the regenerants also

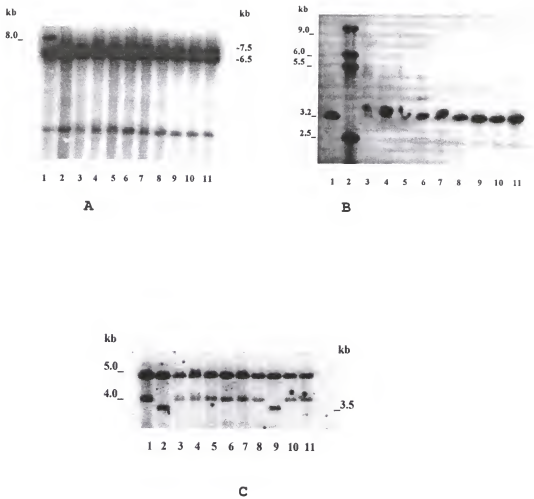


Fig 4.11. Autoradiograph of Southern blot showing genomic DNA (A), mtDNA (B) and chloroplast DNA (C) of the cybrids of 'Willowleaf' (embryogenic parent - lane 1) + 'Valencia' (leaf parent - lane 2). Nine cybrids are shown in lanes 3-11. Enzyme-probe combinations are as follows: panel A, *EcoRI-18SrRNA;* panel B, *HindIII-atpA;* panel C, *BamHI*-cp1.

inherited their chloroplast genome from the embryogenic parent (Fig 4.12).

Cleopatra + Sour Orange

The three seedlings analyzed for this combination were proved cybrids by the genomic *lyc.cyc.* clone and did not present the 8 kb band characteristic of the Cleopatra embryogenic parent. All three cybrids inherited their mitochondrial genome from Cleopatra, without "novel" bands present. Inheritance of the chloroplast genome was again random. One cybrid inherited plastid DNA from Sour Orange (leaf parent) and two inherited it from the embryogenic parent (Fig 4.13).

Summary

The nuclear genome was analyzed in five somatic cybrid combinations where only the leaf parent genome was present. Both somatic hybrids and cybrids presented the embryogenic parent mitochondrial genome, sometimes with nonparental fragments. In contrast, the inheritance of the chloroplast genome in all fusion products was observed to be at random. All results referring to the somatic fusion products are summarized in Tables 4.6 and 4.7.

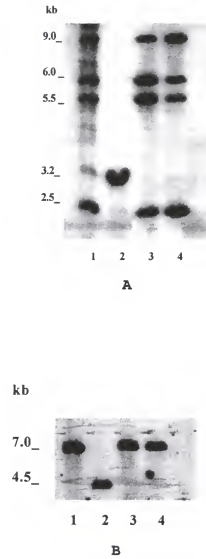


Fig 4.12. Autoradiograph of Southern blot showing mtDNA (A) and chloroplast DNA (B) of the cybrids of 'Hamlin' (embryogenic parent - lane 1) + 'Ponkan' (leaf parent - lane 2). Two cybrids are shown in lanes 3 and 4. Enzyme-probe combinations are as follows: panel A, *HindIII-atpA;* panel B, *PstI*-cp3.

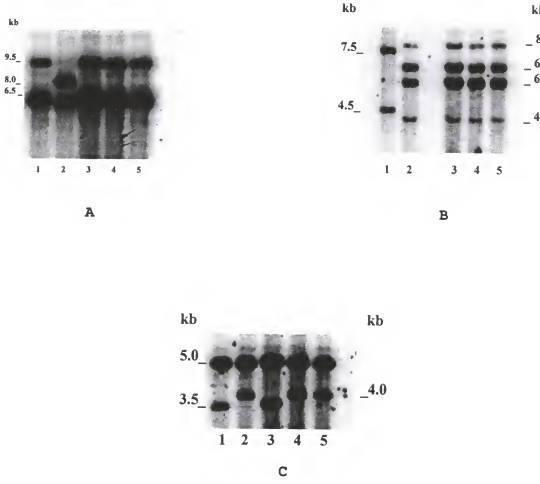


Fig 4.13. Autoradiograph of Southern blot showing genomic DNA (A), mtDNA (B) and chloroplast DNA (C) of the cybrids of Sour Orange (leaf parent - lane 1) + Cleopatra (embryogenic parent - lane 2). Three cybrids are shown in lanes 3-5. Enzyme-probe combinations are as follows: panel A, *EcoRI-18SrRNA;* panel B, *EcoRV-atpA;* panel C, *BamHI*-cp1.

Table 4.6 Summary of Results for the Somatic Hybrids: Genomic and Organelle Analysis.

EMBRYOGENIC PARENT	LEAF PARENT	# [x]	GENOMIC DNA	mtDNA	CHLOROPLAST DNA	NOTES	FIG
'Succari' sweet orange	*Citropsis gilletiana*	14	'Succari' and *C. gilletiana* [y]	'Succari'	'Succari' and *C. gilletiana*	mitochondrial novel bands (1.7/5.5 kb)	4.6
'Succari' sweet orange	*Atalantia ceylanica*	14	'Succari' and *A. ceylanica* [y]	'Succari'	'Succari'	mitochondrial novel bands (4.5/9.0 kb)	4.7

[x] Number of Plants

[y] According to Grosser et al. (1996b)

Table 4.7 Summary of Results for the Somatic Cybrids: Genomic and Organelle Analysis.

EMBRYOGENIC PARENT	LEAF PARENT	# [x]	GENOMIC DNA	mtDNA	CHLOROPLAST DNA	NOTES	FIG
'Willowleaf' mandarin	'Duncan' grapefruit	4	'Duncan'	'Willowleaf'	'Willowleaf' in 1/ 'Duncan' in 3	mitochondrial novel band (9.5 kb)	4.8
Swinglea glutinosa	Sour Orange	3	Sour Orange	*S. glutinosa*[y]	Sour Orange	mitochondrial novel bands (1/2/3/3.5/ 4.5/5.5 kb)	4.9
'Rohde Red Valencia'	'Dancy' mandarin	5	'Dancy'	'Rohde Red Valencia' [y]	'Rohde Red Valencia' in 2/ 'Dancy' in 3	mitochondrial novel band (6.5 kb)	4.10
'Willowleaf' mandarin	'Valencia' sweet orange	9	'Valencia'	'Willowleaf'	'Willowleaf' in 8/ 'Valencia' in 1	No novel mitochondrial bands	4.11
'Hamlin' sweet orange	'Ponkan' mandarin	2	[z]	'Hamlin'	'Hamlin'	No novel mitochondrial bands	4.12
Cleopatra mandarin	Sour Orange	3	Sour Orange	Cleopatra	Cleopatra in 2/Sour Orange in 1	No novel mitochondrial bands	4.13

[x] Number of Plants [y] Results to be discussed [z] No Results Available

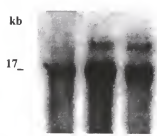
DNA Copy Number Test

The fact that all the somatic hybrids and cybrids inherited their mitochondrial genome primarily from their embryogenic parent may be related, according to Grosser et al. (1996), to the elevated numbers of mitochondria or to the larger amount of mtDNA (C.D.Chase, personal communication) present in suspension cells and callus, in contrast to leaves. This kind of "Copy Number" mechanism will be discussed further in Chapter 5.

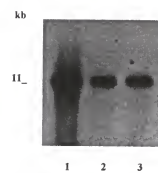
When total DNA from the cybrid parents Sour Orange and Cleopatra (Fig 4.14) was hybridized to the nuclear *lyc.cyc.* clone, bands of about the same intensity could be visualized, independently of the source of DNA material (callus, suspension cells, or leaves). There was good control in terms of the same amount of DNA being loaded in each gel lane. The same results were obtained for the somatic hybrid parents 'Succari' and *Atalantia ceylanica* (Fig 4.15). However, when the same blots were hybridized to the mitochondrial *cob* clone, a distinct difference was observed between the intense band of Cleopatra callus DNA in comparison with a much fainter band in the Cleopatra leaf DNA lane. The difference is even more striking between the 'Succari' suspension DNA and the 'Succari' leaf DNA, demonstrating a greater abundance of mtDNA present in these embryogenic

cultures in comparison with leaves. In contrast, no difference was observed in the relative abundance of chloroplast DNA among leaves, suspension cultures, or callus. This was true for both cybrid and somatic hybrid combinations (Fig 4.14 and 4.15).

Densitometry analysis using the IS-1000 Digital System (Chapter 3) estimated that the amount of mtDNA present in callus of Cleopatra is about 1.5 times the amount present in leaves of this same parent (Fig 4.14B). The same kind of analyses estimated that the amount of mtDNA present in 'Succari' suspension is about 1.7 times the amount present in 'Succari' leaves (Fig 4.15B). Using the same system, no difference was detected in terms of the nuclear and chloroplast genomes, for leaves, suspension or callus DNA.



A



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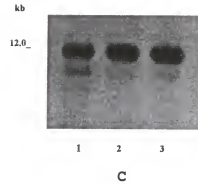

C

Fig 4.14. Autoradiograph of Southern blot showing total DNA of Cleopatra callus (lane 1), Cleopatra leaves (lane 2) and Sour Orange leaves(lane 3), digested with *BamHI* and hybridized with the following probes: panel A, genomic *lyc.cyc.;* panel B, mitochondrial *cob;* panel C, chloroplast *cyt. f.*

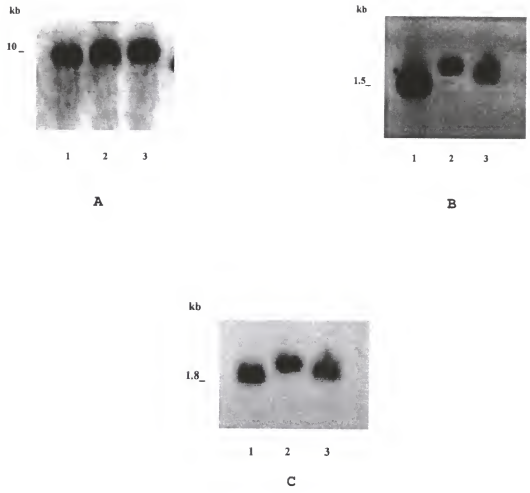

Fig 4.15. Autoradiograph of Southern blot showing total DNA of 'Succari' suspension cells (lane 1), 'Succari' leaves (lane 2) and *Atalantia ceylanica* leaves (lane 3), digested with *EcoRI* and hybridized with the following probes: panel A, genomic *lyc.cyc.;* panel B, mitochondrial *cob;* panel C, chloroplast *cyt. f.*

CHAPTER 5
DISCUSSION

Sexual Cross

Mitochondrial Inheritance

The same seventeen progeny carried the paternal (*P. trifoliata*) configurations for the mitochondrial *atpA*, *coxII* and *coxIII* loci. A possible biparental inheritance of mitochondria in this citrus intergeneric cross was a very surprising result. In contrast to plastids, there are very few reports of deviations from maternal inheritance of mitochondria in angiosperms (Chapter 2). However, as mentioned by Smith (1989), Avise (1991), Milligan (1992), Lee and Taylor (1993), Erickson and Kemble (1993) and Rebould and Zeyl, (1994), the lack of mitochondrial phenotypic markers; the small number of analyzed progeny; and the organelle extraction, DNA isolation, and visualization procedures used in most of the organelle studies, generally are not sensitive enough to detect small amounts of paternal DNA (stoichiometrically underrepresented in hybrid plants) should it be present. During this work, after hybridization with mitochondrial probes, the membranes

were exposed for 7-14 days to a Kodak film. Perhaps, if they had been exposed for fewer days, the paternal configurations would have never been visualized.

However, paternal mtDNA configurations were not observed at all loci. None of the examined progeny carried the *P. trifoliata* RFLPs for the mitochondrial *26rRNA* or *coxI* loci. Perhaps the entire paternal mitochondrial genome was not transmitted or was not subsequently maintained in the progeny. Alternatively, perhaps the original paternal parent (no longer extant) did not carry the *26rRNA* or *coxI* polymorphisms. An intriguing possibility is that there is a nuclear influence over the mitochondrial genome organization in the intergeneric hybrids. The nuclear genome is known to influence the organization of the plant mitochondrial genome, and this will be discussed in the following pages.

Chloroplast Inheritance

Chloroplasts and mitochondria were apparently independently inherited in the sexual cross between LB 1-18 and *P. trifoliata*. Assuming the original paternal parent carried the *P. trifoliata* plastid polymorphisms, a strict maternal inheritance of chloroplasts was observed in the analysis of this citrus intergeneric cross with the *cytf* and *petD* heterologous probes. These data are consistent with the

ideas of Avise (1991), who suggested that there is some active regulation of organelle transmission, since both genomes must be subject to the same physical constraints imposed by the relative sizes of the egg and pollen cytoplasms (Avise, 1991). Tilney-Bassett (1994) has shown that the genetic transmission of plastids is under nuclear control.

Nuclear Influence

Reboud and Zeyl (1994) and Ikehara et al. (1996) indicated that mitochondrial segregation must be controlled by nuclear genes. The single nuclear gene (*Fr*) for the fertility restoration of CMS (cytoplasmic male-sterility) in common bean (Mackenzie and Chase, 1990) and the *chloroplast mutator(chm)* in *Arabidopsis* (Sakamoto et al., 1996), are published examples of single nuclear genes influencing plant mitochondrial genome organization. However, the nuclear influence over the mitochondrial genome organization has been ignored in many previous studies, where the biparental inheritance of organelles has been reported.

Biparental Inheritance: A Hybrid Phenomenon?

The kind of mitochondrial inheritance observed in this sexual cross is consistent with the "receptor-ligand" system

hypothesized by Kaneda et al. (1995) (Chapter 2). These authors observed a paternal mtDNA contribution only in crosses between two different species of mice, but not in intraspecific crosses. Receptor and ligand are mismatched in interspecific hybrids because of the evolutionary divergence, thus preventing efficient recognition and elimination of the sperm mitochondria. This implies the presence of a nuclear control over the mitochondrial inheritance. Hoeh et al. (1991) also agreed that biparental inheritance of mtDNA may be a hybrid phenomenon. Perhaps in the intergeneric cross of LB 1-18 and *Poncirus trifoliata*, "receptor and ligand" are mismatched in these F1 hybrids. In this regard, it would be of interest to examine organelle inheritance in intraspecific crosses of citrus.

Models

Three models were proposed to explain the mitochondrial inheritance pattern observed in the analyzed sexual cross.

1. Simple paternal transmission of mtDNA to some of the progeny.

2. Paternal transmission of mtDNA enabled by nuclear alleles. These nuclear alleles would act in the pollen or in the early embryo, influencing the paternal transmission of mtDNA or the copy number of the paternally transmitted mtDNA

configurations.

3. The influence of segregating nuclear alleles on the maternal mitochondrial genome organization. Potential nuclear influences could generate new configurations by nuclear-gene directed recombination, or even amplify a maternal mitochondrion sublimon (mtDNA configuration present in very low amounts).

Discussion of the First Model

In the first model, the organelle genome is in control of its own inheritance, with no nuclear influence. Using this model, it is difficult to explain why the paternal *coxI* and *26SrRNA* configurations are not present in any of the F1 hybrids, assuming these configurations were present in the male parent. One hypothesis would be that in the way the mitochondrial genome is organized, anything can actually happen. As reviewed by Fauron et al. (1995): "the complex organization and recombinogenic behavior of higher plant mitochondrial genomes make them unique. Because of this high recombination rate, the higher plant mitochondrial genomes show a large diversity of genomic organization between and within species" (p. 234). Recombination between direct repeats could generate minicircles, that could, perhaps, be independently inherited. Perhaps *coxI* and *26SrRNA* are, in

terms of organization of the mitochondrial genome, in different subgenomic circles compared to *atpA*, *coxII* and *coxIII*, and are independently inherited. Recombination can generate this kind of multiple circle organization in the mtDNA (Gillham, 1994).

However, this kind of hypothesis contradicts Birky (1994), in that each organelle chromosome contains a complete set of mitochondrial or chloroplast genes and there is no loss of genes. Since the minicircles do not contain the complete mitochondrial genome, according to this author, every daughter cell must receive at least one mastercircle.

There are still many mysteries concerning plant mtDNA organization. Even its physical structure remains controversial. Although the physical maps present the plant mitochondrial genome as circular molecules, the mtDNA exists more like a large linear and branched molecule *in vivo* (Reviewed by Fauron et al., 1995).

To assess further whether *P. trifoliata* mtDNA configurations are transmitted paternally, a backcross (BC) progeny could be generated by pollinating LB 1-18 with one of the intergeneric hybrids that carry the paternal mtDNA configuration (C.D.Chase, personal communication). The BC progeny would be analyzed for the *P. trifoliata* configurations. If these paternal configurations are generated from the LB 1-18 mitochondrial genome by the

action of a nuclear allele, the BC progeny will again exhibit the paternal configurations. In contrast, the absence of the *P. trifoliata* configurations in the BC progeny, would provide strong evidence for a paternal transmission of the mitochondrial genome. The BC progeny may not inherit these configurations, because the male parent (the intergeneric hybrid) would carry the *P. trifoliata* configurations in very low abundance.

Indeed, the maternal inheritance of just part of the mitochondrial genome (*26SrRNA* and *coxI*) favors the second and third models, where a nuclear allele plays a role in the mitochondrial genome organization.

Discussion of the Second and Third Models

In the second and third models, a nuclear allele is responsible for the inheritance of the paternal configurations (model #2) or the reorganization of the maternal mitochondrial genome (model #3). In considering these models, it is important to remember that *Citrus* plants are usually heterozygous at multiple loci, and this intergeneric progeny would constitute a segregating population with respect to nuclear alleles at multiple loci. The ideal situation to support either of these 2 models would be if these data fit a 1:1 segregation of intergeneric

hybrid progeny with and without *P. trifoliata* mtDNA configurations. However, in this research, 17 hybrids in 26 presented the paternal configurations (X^2 =3.0), indicating deviation from expected 1:1 ratio at *P*=0.05 level of significance. Durham et al. (1992) observed 29/37% of the RFLP markers exhibited skewed segregation in citrus sexual crosses. According to these authors, this is not surprising since deviations from expected ratios have been reported, especially in wide crosses as the one analyzed in this work. Durham et al. (1992) reviewed Zamir and Tadmore (1986) who suggested that such skewed segregation may result from linkage to genes exposed to directional selection at either pre- or post-zygotic stages of development. This skewedness is not thought to result from pleiotropy or epistasis, but to reflect abnormal segregation. Therefore the 1:1 segregation of a nuclear locus is not necessarily expected.

The bulked segregant analysis of RAPD markers would be the most efficient method to determine if a nuclear allele co-segregates with the paternal configurations in these intergeneric hybrids (C.D.Chase, personal communication). RAPD primers could be used to differentiate between two pooled DNA samples: one sample with the seventeen intergeneric hybrids carrying the *P. trifoliata* configurations, and the second with the nine intergeneric hybrids without the paternal mitochondrial RFLPs.

The third model is in part supported by the data presented in Fig. 4.1 (Chapter 4). The paternal *atp9* configurations are observed in the maternal mitochondrial genome, but in very low abundance. Therefore, perhaps what looks like a paternal mitochondrial configuration in some of the F1 hybrids is, in reality, the amplification of sequences already present in the maternal genome. Amplification of substoichiometric sequences has been suggested previously to explain some changes in mtDNA organization (Morgens et al., 1984, reviewed by Small et al., 1987). Small et al. (1989) also suggested that nuclear genes influence the relative copy number of the various mitochondrial genome configurations, although the processes driving these changes are still unknown.

A PCR approach could be used to verify the presence of *atpA, coxII,* and *coxIII P. trifoliata* sequences, at some location in the maternal mitochondrial genome (C.D.Chase, personal communication). If the LB 1-18 mitochondrial genome is arranged such that all of the *P. trifoliata* configurations could be generated by a single recombination event, this would really favor the third model. However, PCR amplification of *P. trifoliata* mtDNA configurations from LB 1-18 mtDNA templates would not preclude paternal transmission as the source of these paternal configurations in the mitochondrial genome of these intergeneric hybrids.

Segregation of the Paternal Configurations

In this research, the segregation of these paternal configurations in a future generation was observed when two of these F1s (# 33 and 44, Fig 4.7 and 4.8) presenting the *P. trifoliata* configurations were outcrossed. Part of these F1s progeny still present the paternal configurations. As in the intergeneric hybrids, the *P. trifoliata* configurations were reduced in copy number relative to the LB 1-18 configurations, but showed a level of abundance similar to that of the F1 maternal parent.

The fate of a heteroplasmic state has been discussed extensively in the literature. According to Hastings (1992), through mitotic segregation the future cytotype would contain exclusively the paternal or maternal configurations. This author sees the heteroplasmic situation presented in this work, as a paternal "leakage", a transitory phenomenon which nuclear alleles would be in charge to reduce. Koehler et al. (1991) documented the replacement of a bovine mtDNA by a sequence variant within one generation. In order to be fixed, this sequence variant must segregate from the majority of maternal mtDNA molecules to become the sole mitochondrial genome in the germ cells of some successive generations. These authors also presented the heteroplasmic state as a transient stage. However, Jazin et al. (1996)

described regions of the human brain where heteroplasmy is a natural state, particularly in noncoding regions.

To assess the consequences of mitochondrial heteroplasmy in the intergeneric hybrid background, C.D.Chase suggested (personal communication) different test-crosses that could be generated using intergeneric hybrids as seed parents. Pollen parents would include *P. trifoliata, C. reticulata* cv. 'Clementine' and *C. paradisi* cv. 'Duncan'. There are a number of possible outcomes for the testcross progeny. If the presence and copy number of *P. trifoliata* mtDNA configurations are strictly regulated by the nuclear genotype, plants co-segregating for these configurations will be recovered. In the progeny presenting the paternal configuration, the relative copy number of the *P. trifoliata* and LB 1-18 configurations should be similar to that of the maternal parent. However, if the *P. trifoliata* mtDNA configurations are stable after being formed, regardless of subsequent changes in the nuclear genotype, the whole progeny would have a similar stoichiometry (no segregation). If organelle sorting out occurs through the female germline, the test cross progeny may exhibit a wide range of phenotypes for the relative copy number of the maternal and paternal configurations. These findings should have important implications in terms of understanding the mechanisms governing the organization and stability of plant

organelle genomes, and the consequences of these mechanisms for plant breeding, genetics and evolution of natural populations.

Somatic Hybrids

MtDNA from the Embryogenic Parent

All the cybrids and somatic hybrids in this work inherited their mitochondrial genome from the embryogenic parent. In some of the combinations, some novel (non-parental) bands were observed among the mitochondrial configurations of these cybrids and somatic hybrids. The presence of these extra bands was also documented by Kumar and Cocking (1987) and reviewed by Smith (1989).

There are different explanations in the literature for the possible origin of these novel bands. Gillham (1994) mentioned that the presence of these novel bands can be due to a preferential amplification and reassortment of "sublimons" already present in low abundance in the intact parents, rather than intergenomic recombination. Tissue culture manipulations can promote extensive alterations in plant mtDNA. Earle (1995) added that mtDNA changes seen in somatic hybrids, or cybrids, might result from changes occurring during the callus or cell suspension stages ("spontaneous culture-related changes"). Earle (1995)

indicated that, in the work of Morgan and Maliga (1987) and Honda et al. (1991), such mtDNA rearrangements were observed in callus of the unfused protoplasts, and not in the leaves. However, Nagy et al. (1983) and Sakai and Imamura (1992), both reviewed by Earle (1995), observed mtDNA changes only in plants derived from fusions, not in plants regenerated directly from protoplast or callus culture of similar protoplasts. Rothenberg and Hanson (1988), reviewed by Earle (1995), identified some of these rearrangement regions and provided some information about how the novel fragments were produced (Chapter 2).

In Fig 4.6 (Chapter 4), an apparent "novel" 6.5 kb mitochondrial *atpA* fragment was present in all cybrids of 'Rohde Red Valencia' + 'Dancy'. However, the same fragment could be visualized in very low amounts in the 'Dancy' (leaf parent) mitochondrial genome. Perhaps, this 6.5 kb fragment is also present in the 'Rohde Red Valencia' mitochondrial configuration, but could not be visualized in the autoradiograph. Instead of being "novel", this fragment could be, in reality, a product of amplification of a sublimon present in the parental mitochondrial genome. This kind of preferential amplification of mtDNA fragments in somatic hybrids was documented in Gramineae by Ozias-Akins et al. (1988). In each somatic hybrid, these authors observed "novel" fragments that were found to be present at

low copy number in one or both of the parental mtDNA, and were amplified 15-30 times in the hybrids.

When checking the 'Succari' + *Citropsis gilletiana* combination with the mitochondrial *atpA* clone, the "novel" bands (1.7 kb) present in the somatic hybrids did not find any match in the mtDNA of 'Succari' suspension cells (Fig 5.1). Therefore, at least for this combination, the appearance of extra bands in the fusion products is probably linked to post-fusion mtDNA rearrangements.

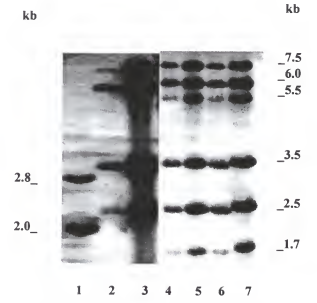

Fig 5.1. Autoradiograph of Southern blot showing mtDNA of *Citropsis gilletiana* leaves (lane 1), 'Succari' leaves (lane 2) and 'Succari' suspension cells (lane 3). Four somatic hybrids that presented the "novel" bands are shown in lanes 4-7. Enzyme-probe combination: *PstI-atpA*.

It is important to remember that, after protoplast fusion, a completely different nuclear composition is formed, and the new nuclear genome can also influence the

organelle genome organization in these somatic hybrids and cybrids. Probably the unique nuclear condition of the new fusion product can influence which mechanism will contribute to variation in the progeny.

Random Chloroplast Inheritance

According to Earle (1995) chloroplasts and mitochondria segregate independently after the somatic fusion. In this research, a completely random inheritance of the chloroplast genome was observed in the somatic hybrids and cybrids: some inherited the plastid genome from the leaf parent, some from the embryogenic parent, and some from both. No novel chloroplast bands were present in any of the cybrids and somatic hybrids. In contrast to mitochondria, Malone et al. (1992) and Earle (1995) mentioned that recombination of chloroplast DNA after fusion is very rare. This work's results for the Cleopatra + Sour Orange combination are in agreement with Grosser et al. (1996). These researches observed the embryogenic parent mtDNA being transmitted to all cybrids and a random chloroplast genome inheritance.

The fact that the 'Succari' + *C. gilletiana* somatic hybrids presented both parental chloroplast genomes (Fig 4.6), is not in agreement with Kumar and Cocking (1987) and Samolylov et al. (1996) who pointed out that although the

fusion products initially contained a mixed population of parental chloroplasts, somatic hybrid/cybrid plants subsequently recovered, in most cases, possess only one or the other parental chloroplast type. When analyzing different citrus somatic hybrids, Kobayashi et al. (1991) also indicated that the chloroplast genome of either one or the other parent was inherited. During this research, all the somatic hybrids and cybrids were analyzed in the very young stage. It would be very interesting to check the same somatic hybrids of 'Succari' + *C. gilletiana*, in a more mature stage, for a possible chloroplast sorting out.

DNA Copy Number Test

According to Grosser et al. (1996), elevated numbers of mitochondria present in the embryogenic cell culture is the reason for the embryogenic parent to be the source of mtDNA for somatic hybrids and cybrids. Bonnema et al. (1992) also pointed out that when suspension cells are the source of protoplasts (with cells growing heterotrophically), this might result in a more competitive form of mitochondria. According to Earle (1995), the somatic hybrids would preferentially inherit the mesophyll parent chloroplast DNA due to the fact that leaf mesophyll cells are relatively enriched in plastids, when compared to suspension-cultured cells. Kumar and Cocking (1987) also

observed a random pattern of inheritance of chloroplast DNA in the symmetric somatic hybrids. However, these authors indicated that mesophyll protoplasts have many more chloroplasts/cell than cell suspension protoplasts and "the prediction would be that the somatic hybrids would preferentially inherit the mesophyll parent chloroplast DNA" (p. 1295).

It is important to note that these authors hypothesized regarding the number of organelles present in different sources of protoplasts. However, there is not a straight correlation between the number of organelles and the amount of organelle DNA in a cell. Calculations of organelle DNA molecules per organelle and per nucleoid reveal that the numbers are quite variable and stage dependent. Metabolically active organelles, for example, have greater numbers of DNA molecules than inactive or undifferentiated organelles. The "unit of inheritance" is still to be characterized, since organelle segregation may occur at the level of organelle DNA molecule, the nucleoid, or the organelle itself. According to Gillham (1994), it is unlikely that a single mechanism can be involved.

In this research, the amount of organelle DNA and not the number of organelles was evaluated. The amount of mtDNA present in the callus and suspension culture was much higher (about 1.7 times) than the amount present in the leaves

(Fig. 4.14 and 4.15). This was true for both, somatic hybrid and cybrid combinations. In contrast, there was no observed variation in the amount of plastid DNA present in leaves, suspension cells or callus cultures (Fig. 4.14 and 4.15). This fact would explain why the plastid DNA was inherited completely at random. Considering this, perhaps the abundance of DNA and not the number of organelles is the important feature in terms of determining the source of organelle DNA for somatic hybrids and cybrids.

Cybrids As Unexpected Outcome

Cybrids as well as somatic hybrids were produced following protoplast fusion of 'Willowleaf' + 'Duncan', *Swinglea glutinosa* + Sour Orange, 'Rohde Red Valencia' + 'Dancy', 'Willowleaf' + 'Valencia', 'Hamlin' + 'Ponkan' and Cleopatra + Sour Orange. The diploid plants with the leaf parent morphology were an unexpected outcome. According to Grosser et al. (1996), these cybrids probably originated from successful protoplast fusion accompanied by failed nuclear fusion and the subsequent loss of the nucleus of the embryogenic parent. Another possibility, mentioned by the same authors, is that mitochondria released from ruptured embryogenic cells got incorporated into mesophyll protoplasts.

The simple fact that the analyzed cybrids are able to

regenerate, is *per se* surprising. In theory, the leaf mesophyll cell would be non-totipotent, eliminating the possibility of whole plant recovery from this parent (Grosser and Gmitter, 1990). However, all the cybrids analyzed in this work present the genomic RFLPs of the leaf parent (although this observation cannot rule out a small genomic contribution from the embryogenic parent, not visualized during these procedures). These results are supported by Saito et al. (1993), whose hypothesis is that possibly the mitochondria of nucellar cells play a significant role in *Citrus* embryogenesis. Further investigation is necessary to determine if this contribution has to do with elevated numbers of mitochondria (Grosser et al., 1996) or with the higher amount of mtDNA present in embryogenic cell cultures, observed in this research. The fact that the plastid DNA in the cybrids was inherited in a random way, agrees with Grosser et al. (1996), that the acquisition of the chloroplast genome from the embryogenic parent is not required for the plant recovery via somatic embryogenesis.

Cybrids Definitions

In reality, what was observed with the analyzed cybrids does not match any of the cybrids descriptions reviewed in the literature (Chapter 2). The cybrids obtained by Vardi et

al. (1989) using the "Donor-Recipient" Method (Chapter 2) present the nuclear genome from one parent and the organelle genomes from the other parent. In the cybrid description presented by Bonnema et al. (1992) and Kumar and Cocking (1987), the rule would be that the organelle genome would be inherited from both parents. In the analyzed cybrids, no rule was observed in terms of the plastid genome. Only in terms of the nuclei and mitochondrial genome, we could establish fixed parameters.

According to Grosser et al. (1996), the practical value of citrus cybrids is currently unknown. However, the fact that new combinations of nuclei and organelles can be obtained after protoplast fusion, may have interesting applications to the creation of novel plant materials of potential horticulture value.

CHAPTER 6
CONCLUSIONS

Sexual Cross

The results of this research permit the following conclusions:

a) Chloroplasts and mitochondria were independently inherited in the 26 sexual hybrids between LB 1-18 and *Poncirus trifoliata*.

b) There was transmission of the mitochondrial *atpA, coxII* and *coxIII* paternal configurations in 17 of the 26 sexual hybrids of LB 1-18 x *P. trifoliata*. In contrast, the mitochondrial *coxI* and *26SrRNA* were maternally inherited in all the 26 sexual hybrids. This intriguing fact raised the hypothesis that the nuclear genome plays an important role in the mitochondrial genome organization. The nuclear influence over the mitochondrial genome has been ignored in previous studies, where the biparental inheritance of organelles was documented.

c) There was transmission of the paternal mtDNA configurations to the outcross progeny of 2 of the F1

hybrids.

d) A strict maternal inheritance of the chloroplast *cyt.f* and *petD* was observed in this citrus intergeneric cross.

Somatic Fusions

a) Chloroplasts and mitochondria segregate independently after the somatic fusions.

b) The genomic *lyc.cyc.* and *18SrRNA* clones were useful to prove the somatic cybrid nature in 5 of the 8 analyzed somatic fusions.

c) The somatic hybrids presented the nuclear genome of both parents. In the cybrids, only the leaf parent's nuclear genome was observed.

d) The cybrids and somatic hybrids in this work inherited their mitochondrial genome from the embryogenic parent. However, in some combinations, "novel" (non parental) bands were observed among the mitochondrial configurations of these cybrids and somatic hybrids. For some fusions, this resulted in difficulties of interpretation in the results concerning the mitochondrial genome.

e) The appearance of "novel" bands in the fusion products of one somatic cybrid combination was linked to

post-fusion mtDNA rearrangements - the amplification of parental sublimons or recombination between the parental mitochondrial genomes.

f) There was a random chloroplast genome inheritance in all the somatic fusion products. Some inherited the plastid genome from the leaf parent, some from the embryogenic parent, and some from both. No novel chloroplast bands were present in any of the cybrids and somatic hybrids.

g) The DNA copy number test proved that mtDNA was more abundant in embryogenic cell cultures than in leaves. This is probably the reason why the embryogenic parent is the source of mtDNA for somatic hybrids and cybrids.

h) In contrast, there was no observed variation in the abundance of plastid DNA present in leaves, suspension cells or callus cultures, according to the same copy number test. This fact would explain why the plastid DNA was inherited completely at random. The copy number of plastids probably did not play a role in determining the source of the chloroplast genome.

REFERENCES

Akagi, H., T. Taguchi, T. Fujimura. 1995. Stable inheritance and expression of the CMS traits introduced by asymmetric protoplast fusion. Theor. Appl. Genet. 91: 563-567.

Allen, J.F. 1996. Separate sexes and the theory of ageing. J. Theor. Biol. 180: 135-140.

Avise, J.C. 1991. Ten unorthodox perspectives on evolution prompted by comparative population genetic findings on mitochondrial DNA. Ann. Rev. Genet. 25: 45-69.

Berger, K.H., M.P. Yaffe. 1996. Mitochondrial distribution and inheritance. Experimentia 52: 1111-1116.

Birky, C.W. Jr. 1994. Relaxed and stringent genomes: why cytoplasmic genes don't obey Mendel's laws. J. Hered. 85: 355-365.

Bonnema, A.B., J.M. Melzer, L.W. Murray, O.C. MA. 1992. Non-random inheritance of organellar genomes in symmetric and asymmetric somatic hybrids between *Lycopersicon esculentum* and *L. pennellii*. Theor. Appl. Genet. 84: 435-442.

Chase, C.D., V.M. Ortega. 1992. Organization of ATPA coding and 3'flanking sequences associated with cytoplasmic male sterility in *Phaseolus vulgaris* L. Curr. Genet. 22: 147-153.

Chiu, W-L., B. Sears. 1993. Plastome-genome interactions affect plastid transmission in *Oenothera*. Genetics 133: 989-997.

Chiu, W.L., W. Stubbe, B.B. Sears. 1988. Plastid inheritance in Oenothera: organelle genome modifies the extent of biparental plastid transmission. Curr. Genet. 13: 181-189.

Cipriani, G., R. Testolin, M. Morgante. 1995. Paternal inheritance of plastids in interspecific hybrids of the genus *Actinidia* revealed by PCR amplification of chloroplast DNA fragments. Mol. Gen. Genet. 247: 693-697.

Davies, F.S., L.G. Albrigo. 1994. Citrus, 1st ed. CAB International, Trowbridge, UK, 254 pp.

Dawson, A.J., V.P. Jones, C.J. Leaver. 1984. The apocytochrome b gene in maize mitochondria does not contain introns and is preceded by a potential ribosome binding site. The EMBO Journal 3 (9): 2107-2113.

Durham, R.E., P.C. Liou, F.G. Gmitter Jr, G.A. Moore. 1992. Linkage of restriction fragment length polymorphisms and isozymes in *Citrus*. Theor. Appl. Genet. 84: 39-48.

Earle, E.D. 1995. Mitochondrial DNA in somatic hybrids and cybrids, vol. 3. Kluwer Academic Publishers, Boston, pp. 557-584.

Ecke, W., G. Pannenbecker, O. Wasmund, G. Michaelis. 1989. Extranuclear inheritance: mitochondrial genetics. Progress in Botany 50: 198-206.

Eckenrode, V.K., J. Arnold, R.B. Meagher. 1985. Comparison of the Nucleotide Sequence of Soybean 18S rRNA with the Sequences of other Small-Subunit rRNAs. J. Mol. Evol. 21: 259-269

Erickson, L., R. Kemble. 1990. Paternal inheritance of mitochondria in rapeseed (*Brassica napus*). Mol. Gen. Genet. 222: 135-139.

Erickson, L., R. Kemble. 1993. The effect of genotype on pollen transmission of mitochondria in rapeseed (*Brassica napus*). Sexual Plant Reproduction 6: 33-39.

Fairbanks, D.J., S.E. Smith, J.K. Brown. 1988. Inheritance of large mitochondrial RNA's in alfalfa. Theor. Appl. Genet. 76: 619-622.

Faure, S., J.L. Noyer, F. Carreel, J.P. Horry, F. Bakry, C. Lanaud. 1994. Maternal inheritance of chloroplast genome and paternal inheritance of mitochondrial genome in bananas (*Musa acuminata*). Curr. Genet. 25: 265-269.

Fauron, C., M. Casper, Y. Gao, B. Moore. 1995. The maize mitochondrial genome: dynamic, yet functional. TIG 11: 228-235.

Feinberg, A., B. Volgestein. 1984. A technique for radiolabeling DNA restriction fragments to high specific activity. Anal. Biochem. 137: 266-267.

Fox, T.D., C.J. Leaver. 1981. The *Zea mays* mitochondrial gene coding cytochrome oxidase subunit II has an intervening sequence and does not contain TGA codons. Cell 26: 315-323.

Gillham, N.W. 1994. Organelle genes and genomes. Oxford University Press, New York, 424 pp.

Gmitter, F.G. Jr., S.Y. Xiao, S. Huang, X.L. Hu, S.M. Garnsey, Z. Deng. 1996. A localized linkage map of the citrus tristeza virus resistance gene region. Theor. Appl. Genet. 92: 688-695.

Gotoh, T., H. Oku, K. Moriya, M. Odawara. 1995. Nucleus-cytoplasm interactions causing reproductive incompatibility between two populations of *Tetranychus quercivorus* Ehara et Gotoh (Acari: Tetranychidae). Heredity 74: 405-414.

Grosser, J.W., F.G. Gmitter Jr. 1990. Protoplast fusion and citrus improvement. Plant Breeding Reviews 8: 339-374.

Grosser, J.W., F.G. Gmitter Jr, N. Tusa, G.R. Recupero, P. Cucinotta. 1996. Further evidence of a cybridization requirement for plant regeneration from citrus leaf protoplasts following somatic fusion. Plant Cell Reports 15: 672-676.

Grosser, J.W., F.A.A. Mourao-Fo, F.G. Gmitter Jr, E.S. Louzada, J. Jiang, K. Baergen, A. Quiros, C. Cabasson, J.L. Schell, J.L. Chandler. 1996b. Allotetraploid hybrids between *Citrus* and seven related genera produced by somatic hybridization. Theor. Appl. Genet. 92: 577-582.

Guo, F.L., S.Y. Hu. 1995. Cytological evidence of biparental inheritance of plastids and mitochondria in Pelargonium. Protoplasma 186: 201-207.

Hastings, I.M. 1992. Population genetic aspects of deleterious cytoplasmic genomes and their effect on the

evolution of sexual reproduction. Genetical Research 59: 215-225.

Hause, G. 1986. Organelle distribution during pollen development of Pisum sativum L. Biologisches Zentralblatt 105: 283-288.

Havey, M.J. 1997. Predominant paternal transmission of the mitochondria genome in cucumber. J. Hered. 88: 232-235.

Hoeh, W.R., K.H. Blakley, W.M. Brown. 1991. Heteroplasmy suggests limited biparental inheritance in *Mytilus* mitochondrial DNA. Science 251: 1488-1490.

Honda , H., A. Hirai. 1992. The gene for the alpha-subunit of the ATPase: a site of homologous recombination in plant mitochondrial DNA also functions in somatic hybrid cells. Theor. Appl. Genet. 84: 33-38.

Ikehara, T., L. Suzuki, S. Nakamura. 1996. Chloroplast nucleoids in large number and large DNA amount with regard to maternal inheritance in *Chlamydomonas reinhardtii*. Protoplasma 194: 11-17.

Isaac, P.G., V.P. Jones, C.J. Leaver. 1985. The maize cytochrome c oxidase subunit I gene: sequence , expression and rearrangement in cytoplasm male sterile plants. The EMBO Journal 14 (7): 1617-1623

Jazin, E.E., L. Cavelier, I. Eriksson, L. Oreland, U. Gyllenstein. 1996. Human brain contains high levels of heteroplasmy in the noncoding regions of mitochondrial DNA. Proc. Natl. Acad. Sci. USA 93: 12382-12387.

Kaneda, H., J. Hayashi, S. Takahama, C. Taya, K.F. Lindahl, H. Yonekawa. 1995. Elimination of paternal mitochondria DNA in intraspecific crosses during early mouse embryogenesis. Proc. Natl. Acad. Sci. USA 92: 4542-4546.

Kiang, A.S., V. Connolly, D.J. McConnell, T.A. Kavanagh. 1994. Paternal inheritance of mitochondria and chloroplasts in *Festuca pratensis-Lolium perenne* intergeneric hybrids. Theor. Appl. Genet. 87: 681-688.

Kobayashi, S., T. Ohgawara, K. Fujiwara, I. Oiyama. 1991. Analysis of cytoplasmic genomes in somatic hybrids between

navel orange (*Citrus sinensis* Osb.) and 'Murcott'tangor. Theor. Appl. Genet. 82: 6-10.

Koehler, C.M., G.L. Lindberg, D.R. Brown, D.C. Beitz, A.E. Freeman, J.E. Mayfield, A.M. Myers. 1991. Replacement of bovine mitochondrial DNA by a sequence variant within one generation. Genetics 129: 247-255.

Kondo, R., Y. Satta, E.T. Matsuura, H. Ishiwa, N. Takahata, S.I. Chigusa. 1990. Incomplete maternal transmission of mitochondrial DNA in *Drosophila*. Genetics 126: 657-663.

Kumar, A., E.C. Cocking. 1987. Protoplast fusion: a novel approach to organelle genetics in higher plants. Am. J. Bot. 74: 1289-1303.

Kuroiwa, T., H. Uchida. 1996. Organelle division and cytoplasmic inheritance. BioScience 46: 827-835.

Laipis, P.J., M.J. Van de Walle, W.W. Hauswirth. 1988. Unequal partitioning of bovine mitochondrial genotypes among siblings. Proc. Natl. Acad. Sci. USA 85: 8107-8110.

Lee, D.J., T.K. Blake, S.E. Smith. 1988. Biparental inheritance of chloroplast DNA and the existence of heteroplasmic cells in alfalfa. Theor. Appl. Genet. 76: 545-549.

Lee, S.B., J.W. Taylor. 1993. Uniparental Inheritance and Replacement of Mitochondrial DNA in *Neurospora tetrasperma*. Genetics 134: 1063-1075.

Mackenzie, S.A., C.D. Chase. 1990. Fertility restoration is associated with loss of a portion of the mitochondrial genome in cytoplasmic male sterile common bean. The Plant Cell 2: 905-912.

Mackenzie, S.A., S. He, A. Lyznik. 1994. The elusive plant mitochondrion as a genetic system. Plant. Physiol. 105: 775-780.

Malek, O., K. Lating, R. Hiesel, A. Brennicke, V. Knoop. 1996. RNA editing in bryophites and a molecular phylogeny of land plants. The EMBO Journal 15: 1403-1411.

Malone, R., G.V. Horvath, A. Cseplo, B. Buzas, P.J. Dix, P. Medgeyesy. 1992. Impact of the stringency of cell selection

on plastid segregation in protoplast fusion-derived *Nicotiana* regenerates. Theor. Appl. Genet. 84: 866-871.

Mason, R.J., K.E. Holsinger, R.K. Jansen. 1994. Biparental inheritance of the chloroplast genome in *Coreopsis* (Asteraceae). J. Hered. 85: 171-173.

Matsuura, E.T., S.I. Chigusa, Y. Nike. 1989. Induction of mitochondrial DNA heteroplasmy by intra and interspecific transplantation of germ plasma in *Drosophila*. Genetics 122: 663-667.

Medgyesy,P., E. Fejes, P. Maliga. 1985. Interspecific chloroplast recombination in a *Nicotiana* somatic hybrid. Proc. Natl. Acad. Sci. USA. 82: 6960-6964.

Milligan, B.G. 1992. Is organelle DNA strictly maternally inherited? Power analysis of a binomial distribution. Am. J. Bot. 79: 1325-1328.

Mogensen, H.L. 1988. Exclusion of male mitochondria and plastids during syngamy in barley as a basis for maternal inheritance. Proc. Natl. Acad. Sci. USA 85: 2594-2597.

Mogensen, H.L. 1996. The hows and whys of cytoplasmic inheritance in seed plants. Am. J. Bot. 83: 383-404.

Moon, E., T. Kao, R. Wu. 1985. Pea Cytochrome subunit II gene has no intron and generates two mRNA transcripts with different 5'termini. Nucleic Acids Res. 13: 3195-3205.

Nakamura, S., T. Ikehara, H. Uchida, T. Suzuki, Sodmergen. 1992. Fluorescence microscopy of plastid nucleoids and a survey of nuclease C in higher plants with respect to mode of plastid inheritance. Protoplasma 169: 68-74.

Neale, D.B., K.A. Marshall, R.R. Sederoff. 1989. Chloroplast and mitochondrial DNA are paternally inherited in *Sequoia sempervirens* D. Don Endl. Proc. Natl. Acad. Sci. USA 86: 9347-9349.

Ohgawara, T., H. Uchimya, S. Ishii, S. Kobayashi. 1994. Somatic hybridization between *Citrus sinensis* and *Poncirus trifoliata*. Biotechnology in Agriculture and Forestry vol. 27. Springer-Verlag, Berlin, Germany, pp. 439-454.

Ozias-Akins, P., Z. Tabaeizadeh, D.R. Pring, I.K. Vasil. 1988. Preferential amplification of mitochondrial DNA fragments in somatic hybrids of the Gramineae. Curr. Genet. 13: 241-245.

Palmer, J.D., W.F. Thompson. 1981a. Rearrangements in the chloroplast genomes of mung bean and pea. Proc. Natl. Acad. Sci. USA 78: 5533-5537.

Palmer, J.D., W.F. Thompson. 1981b. Clone banks of the mung bean, pea and spinach chloroplast genomes. Gene 15: 21-26.

Petri, B. 1996. Extreme Sequence Heteroplasmy in Bat Mitochondrial-DNA. Biol. Chem. 377: 661-667.

Pring, D., A. Brennicke, W. Schuster. 1993. RNA editing gives a new meaning to the genetic information in mitochondria and chloroplasts. Plant Mol. Biol. 21: 1163-1170.

Rajora, O.P., D.J. Mahon. 1995. Paternal plastid DNA can be inherited in lentil. Theor. Appl. Genet. 90: 607-610.

Reboud, X., C. Zeyl. 1994. Organelle inheritance in plants. Heredity 72: 132-140.

Russell, P.J. 1992. Genetics, Third ed. Harper Collins Publishers, New York, 758 pp.

Sager, R. 1972. Cytoplasmic Genes and Organelles. Academic Press, New York, 405 pp.

Saito, W., T. Ohgawara, J. Shimizu, S. Ishii, S. Kobayashi. 1993. Citrus cybrid regeneration following cell fusion between nucellar cells and mesophyll cells. Plant Science 88: 195-201.

Sakamoto, W., H. Kondo, M. Murata, F. Motoyoshi. 1996. Altered mitochondrial gene expression in a maternal distorted leaf mutant of Arabidopsis induced by chloroplast mutator. Plant Cell 8: 1377-1390.

Salazar, R.A., D.R. Pring, F. Kempken. 1991. Editing of mitochondrial *atp9* transcripts from two sorghum lines. Curr. Genet. 20: 483-486.

Sambrook, J., E.F. Fristsch, T. Maniatis. 1989. Molecular cloning (a laboratory manual), Second ed, vol. 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1752 pp.

Samoylov, V.M., S. Izhar, K.C. Sink. 1996. Donor chromosome elimination and organelle composition of asymmetric somatic hybrid plants between an interspecific tomato hybrid and eggplant. Theor. Appl. Genet. 93: 268-274.

Shore, J.S., K.L. McQueen, S.H. Little. 1994. Inheritance of plastid DNA in the *Turnera ulmifolia* complex (Turneraceae). Am. J. Bot. 81: 1636-1639.

Small, I.D., P.J. Isaac, C.J. Leaver. 1987. Stoichiometry differences in DNA molecules containing the atpA gene suggest mechanisms for the generation of mitochondrial genome diversity in maize. The EMBO Journal 6: 865-869.

Small, I., R. Suffolk, C.J. Leaver. 1989. Evolution of plant mitochondrial genomes via substochiometric intermediates. Cell 58: 69-76.

Smith, S.E. 1989. Biparental inheritance of organelles and its implications in crop improvement. Plant Breeding Reviews 6: 361-393.

Sodmergen, Y.Y. Luo, T. Kuroiwa, S.Y. Hu. 1994. Cytoplasmic DNA apportionment and plastid differentiation during male gametophyte development in *Pelargonium zonale*. Sexual Plant Reproduction 7: 51-56.

Solignac, M., M. Monnerot, J-C. Mounolou. 1983. Mitochondria DNA heteroplasmy in *Drosophila mauritiana*. Proc. Natl. Acad. Sci. USA 80: 6942-6946.

Soliman, K., G. Fedak, R.W. Allard. 1987. Inheritance of organelle DNA in barley and *Hordeum X Secale* intergeneric hybrids. Genome 29: 867-872.

Stoneking, M. 1996. Mitochondrial DNA heteroplasmy: out of the closet. Biol. Chem. 377: 603-604.

Tilney-Basset, R.A.E. 1994. Nuclear controls of chloroplast inheritance in higher plants. J. Hered. 85: 347-354.

Turner, C.M., G. Hide, N. Buchanan, A. Tait. 1995. *Trypanosoma brucei*: inheritance of kinetoplast DNA maxicircles in a genetic cross and their segregation during vegetative growth. Exp. Parasitol. 80: 234-41.

Vardi, A., P. Arzee-Gonen, A. Frydman-Shani, S. Bleichman, E. Galun. 1989. Protoplast fusion mediated transfer of organelles from *Microcitrus* into *Citrus* and regeneration of novel alloplasmic trees. Theor. Appl. Genet. 78: 741-747.

Vardi, A., A. Breiman, E. Galun. 1987. Citrus cybrids : production by donor-recipient protoplast-fusion and verification by mitochondrial-DNA restriction profiles. Theor. Appl. Genet. 75: 51-58.

Vardi, A., E. Galun, A. Frydman-Shani, P. Conen, S. Bliechman. 1990. Citrus cybrids: transfer of *Microcitrus* organelles into *Citrus* cultivars. Acta Horticulturae 280: 239-245.

Vaughn, K.C. 1981. Organelle transmission in higher plants: organelle alteration vs. physical exclusion. J. Hered. 72: 335-337.

Warren, G., W. Wickner. 1996. Organelle inheritance. Cell 84: 395-400.

Willey, D.L., A.D. Auffret, J.C. Gray. 1984. Structure and topology of cytochrome f in pea chloroplast membranes. Cell 36: 555-562.

Wilson, R.K., M. Hanson. 1996. Preferential RNA editing at specific sites within transcripts of two plant mitochondrial genes does not depend on transcriptional context or nuclear genotype. Curr. Genet. 30: 502-508.

Yamamoto, M., S. Kobayashi. 1995. A cybrid plant produced by eletrofusion between *Citrus unshiu* (satsuma mandarin) and *C. sinensis* (sweet orange). Plant Tissue Culture Letters 12: 131-137.

Yu, H., B. Huang, S. Russell. 1994. Transmission of male cytoplasm during fertilization in *Nicotiana tabacum*. Sex Plant Reprod. 7: 313-323.

Zurowski, P., B. Perot, W. Bottomley, P.R. Whitfeld. 1981. The structure of the gene for the larger subunit of

ribulose 1-5 bisphosphate carboxylase from spinach chloroplast DNA. Nucleic Acids Res. 9: 3251-3270.

BIOGRAPHICAL SKETCH

Maria Cristina Drummond Moreira was born in Sao Paulo, Brazil. She attended elementary, middle and high school there. In 1979, she entered the University of Sao Paulo, from which she received a Bachelor of Science degree in agronomy in 1983. After graduation, she continued her education at the same university obtaining a Master of Science degree in horticultural sciences in 1988. Her research dealt with the use of growth regulators to avoid the pre-harvest drop of citrus fruits. In 1989, she was hired by BIOTECH, a British biotechnology company located in Brazil, to work with tissue culture of tropical and subtropical fruit plants. In 1992, with the financial support of CNPq, she became part of the biotechnology lab of the Citriculture Experimental Station in Limeira, Sao Paulo, Brazil, to work with molecular markers in the taxonomic characterization of Citrus. In 1993, she was accepted by the Horticultural Sciences Department of the University of Florida as a graduate student in a Ph.D. program. She was awarded a scholarship from CNPq for this purpose.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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